Role of *Bmp2* in Dental Hard Tissue Formation

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1. Summary

During embryonic development various organs develop through epithelial-mesenchymal interactions. Few conserved signaling pathways have shown to play an important role in this process. Bone Morphogenetic Protein (Bmp) signaling is an important pathway involved in organogenesis. Bmp antagonists regulate the activity of Bmps in the extracellular space, which controls the quantity and quality of Bmp signals transduced. Most mouse mutants deficient in either Bmp or Bmp antagonists do not survive limiting our understanding of the Bmp pathway in organogenesis.

Tooth development is successfully used as a genetic model to study organs that arise through epithelial- mesenchymal interactions. We utilized it as a model to study the role of *Bmp2* during tooth development with a focus on epithelial- mesenchymal interaction and understanding the early stages of dental hard tissue formation. We studied *Bmp2* deletion in epithelium by using K-14 Cre and *Bmp2* deletion in mesenchyme using Wnt-1 Cre. Epithelial deletion of *Bmp2* showed minor enamel defects in incisors along with unorganized Stratum intermedium (SI) layer indicating its importance in the underlying SI layer for enamel formation. *Bmp2* deletion in mesenchyme resulted in odontoblast, dentin and enamel defects. The molecular mechanisms controlling tooth mineralization were analyzed in more detail in newborn pups (P0), the time point when the *Bmp2* fl/fl;Wnt1-Cre mutant mice died. Loss of *Bmp2* decreased Bmp signaling in general and led to reduction of several key molecules involved in dentin matrix and enamel formation. RNA seq on isolated P0 molar teeth confirmed the overall decrease in various epithelial and mesenchymal genes in mutants suggesting role of *Bmp2* in reciprocal epithelial-mesenchymal interactions. This is exemplified by reduced epithelial expression of *Pax9*, a transcription factor with predominant mesenchymal expression in early tooth development that has been associated with oligodontia. Interestingly, several Wnt pathway genes were also downregulated leading to an overall decrease in Wnt signaling in mutant teeth. These data indicate that *Bmp2* is an important regulator for Wnt-mediated odontobast differentiation and dentin formation.

To study the consequences of *Bmp2* deletion in adult teeth, embryonic tooth germs were ectopically transplanted under kidney capsules of the recipient mice (*Bmp2* fl/fl) and allowed to mature for 4 weeks. Adult mutant teeth showed pulp obliteration and pulp calcification. An overall reduction of Bmp signaling was found in adult teeth including periodontium, roots, and alveolar bone.

In summary, this study establishes that *Bmp2* is involved in epithelial-mesenchymal interactions during tooth mineralization stages, that loss of *Bmp2* leads to odontoblast, dentin, ameloblasts, and enamel defects in part by direct modulation of transcription factors, mineral matrix formation, tissue calcification, as well as Wnt signaling. Loss of *Bmp2* in adult teeth results in disturbed pulp and odontoblast homeostasis associated with ectopic deposition of osteodentin reminiscent of dentinogenesis imperfecta.

2. Summary in German

Verschiedene Organe entwickeln sich während der Embyronalentwicklung durch epitheliale-mesenchymale Interaktionen. Einige wenige konservierte Signalmoleküle und ihre Signaltransduktionswege spielen bei diesem Prozess eine wichtige Rolle. Knochen Morphogenetische Proteine (Bone Morphogenetic Proteins, BMPs) gehören zu dieser Klasse und sind Signal-Moleküle, die weitverbreitet in der Organogenese involviert sind. Ihre Aktivität wird im extrazellulären Bereich durch BMP Antagonisten reguliert, welche Menge und Quantität des transduzierten BMP Signals kontrollieren. Mausmutanten, denen BMP oder BMP Antagonisten fehlen, überleben häufig nicht bis zur Geburt. Dies limitiert unser Verständnis von der Rolle des BMP Signalweges in der Organentwicklung.

Zahnentwicklung wird erfolgreich als genetisches Model für Organe benutzt, die sich durch epitheliale-mesenchymale Interaktionen entwickeln. Wir haben sie als Model für die Rolle von BMP2 während der Zahnentwicklung benutzt und dabei auf epitheliale-mesenchymale Interaktionen und das Verständnis der frühen Zahnhartsubstanzbildung fokusiert. Da Zähne sich durch epitheliale-mesenchymale Interaktionen entwickeln, haben wir die Konsequenzen der *Bmp2*-Elimination im Epithel mittels Keratin14-Cre und im Mesenchyme mittels Wnt1-Cre untersucht. Elimination im Epithel hatte geringe Zahnschmelzdefekte in Schneidezähnen zur Folge, begleitet von einem unorganisiertem Stratum Intermedium (SI), was die seine Wichtigkeit für die Schmelzbildung unterstreicht. Elimination von *Bmp2* im Mesenchyme führte zu Odontoblasten, Dentin und Schmelzdefekten. Daraufhin wurden die molekularen Mechanismen, welche die Zahnmineralisation kontrollieren, detaillierter in Neugeborenen (P0), die zu diesem Zeitpunkt versterben, untersucht. Bmp2-Verlust hatte generell verminderte BMP-Signaltransduktion sowie verminderte Expression mehrerer Schlüsselmoleküle für Dentinmatrix und Schmelzbildung zur Folge. RNAseq Analyse von isolierten P0 Molaren bestätigte die gesamthaft verminderte Expression verschiedener epithelialer oder mesenchymaler Gene in Mutanten, was eine Rolle von Bmp2 in epithelialen-mesenchymalen Interaktionen nahelegt. Zur Illustration, die Expression des

Transkriptionsfaktors Pax9, der während der frühen Zahnentwicklung vornehmlich mesenchymal exprimiert und mit Oligodontia assoziiert ist, war im Epithel stark reduziert. Interessanterweise waren mehrere Gene des Wnt-Signaltransduktinosweges reduziert, was zu einer gesamthaften Reduktion von Wnt-Signalen in Zahnmutanten führte. Diese Daten zeigen, dass *Bmp2* ein wichtiger Regulator für Wnt-vermittelte Odontoblastendifferenzierung und Dentinbildung ist. Um die Konsequenzen der Bmp2-Elimination in erwachsenen Zähnen zu untersuchen, wurden embryonale Zahnkeime unter die Nierenkapsel von Empfängermäusen transplantiert und während vier Wochen reifen gelassen. Solche erwachsenen Zahnmutanten zeigten Obliteration und Kalzifizierung der Zahnpulpa. Interessanterweise wurde auch eine generelle Reduktion von Bmp-Signalen in Parodont, Wurzel und Alveolarknochen solcher Mutanten festgestellt.

Zusammenfassend zeigt diese Studie, dass Bmp2 in epithelialen-mesenchymalen Interaktionen während der Zahnmineralisationsstadien involviert ist und dass Verlust von Bmp2 mit Odontoblasten-, Dentin-, Ameloblasten- und Schmelz-Defekten einhergeht, welche durch teilweise direkte Modulation von Transkriptionsfaktoren, Mineralmatrixbildung, Gewebekalzifizierung sowie Wnt-Signalübertragung verursacht wurden. Bmp2-Verlust in erwachsenen Zähnen führte zu gestörter der Zahnpulpa- und Odontoblasten Homöostase, was mit ektopischer Osteodentindeposition, ähnlich wie bei Dentinogenesis Imperfecta, assoziiert war.

3. Introduction

Appropriate cell-cell interactions mediated through signal transduction pathways are crucial for development of an embryo. Despite a wide variety of cell types and tissue structures, only a few conserved signaling pathways have shown to be involved in embryonic development, which include Bmps (Lan et al., 2014). Bmps are secreted proteins involved in a wide variety of tissues during embryogenesis and tissue homeostasis (Brazil et al., 2015a). Many biochemical and genetic studies performed have dissected the molecular components of the Bmp pathway, but how Bmp signaling is modulated in organogenesis and developmental patterning is still poorly understood.

Teeth like many organs arise through reciprocal epithelial-mesenchymal interactions. Teeth serve as an excellent model for experimental manipulation in understanding developmental patterning during organogenesis (Pispa and Thesleff, 2003)(Thesleff and Sharpe, 1997). For example- isolated tooth germs, even dissociated or recombined can develop into mineralized tooth upon ectopic transplantation in renal capsule in mice (Jernvall and Thesleff, 2000; Tucker and Sharpe, 2004). In this project we used tooth as a model to study how Bmp signaling regulates epithelial-mesenchymal interactions during development. In particular, we sought to understand the role of *Bmp2* at the early mineralization stages of tooth development.

3.1 Bone morphogenetic proteins (Bmps)

Bmps are evolutionary conserved secreted signaling proteins, which belong to the TGF-β family. Though initially discovered as bone inducing factors, now they are known to be involved in a wide array of biological processes and structures, such as gastrulation, organ development, tissue homeostasis and repair, and cancer (Wagner et al., 2010). More than 22 members of the Bmp family have been identified. Synthesis and secretion of Bmp ligands occurs as larger propeptides form, which is cleaved by extracellular proprotein convertases

such as Furins (Cui et al., 1998). Mature Bmp ligands bind to BmprI/II and form hexameric complexes (Figure 3.1). Signaling is initiated by binding to type I Bmp receptors (BmprI) [ALK 2,3 or 6 (Activin like Kinases)] which then binds to type II Bmp receptors (BmprII). This phosphorylates the GS repeat domain of BmprI (Bagarova et al., 2013; Kaplan et al., 2009). Once activated BmprI phosphorylates a set of Smad proteins called receptor Smads 1, 5, 8 (R-Smad 1/5/8), which in turn bind to Smad4. This complex translocates to, and is accumulated, in the nucleus where it directs transcription of target genes by binding to Bmp responsive elements (Miyazono et al., 2005)(Figure 3.1).

Bmp signaling is regulated at various levels. In the intracellular space regulatory proteins control Bmp signaling downstream of Bmp receptor activation (Figure 3.1). Negative regulation of Bmp signaling is by inhibitory Smads (Smad 6 and Smad7) and miRNAs (Brazil et al., 2015a). In the extracellular space Bmp regulation occurs by pseudoreceptors such as BAMBI and Bmp antagonists (Figure 3.2), which control the quantity and quality of Bmp signals transduced (Walsh et al., 2010). Recent studies have shown that a cross-talk exists between Bmp and other signaling pathways such as Wnt, Tgfβ and Hedgehog. For example Bmp2 signalling in epicardial cells is dependent on Tgfβ receptor type3 (Kirkbride et al., 2008). Wnt3a activation or β-catenin/TCF4 activation activates Bmp2 expression in osteoblasts (Rongrong Zhang et al., 2013). Also, disheveled/Par1b can facilitate Tgfβ signaling in mammalian HEK293 cells and Xenopus mesoderm development (Mamidi et al., 2012). Each step in Bmp pathway is tightly regulated emphasizing the critical nature of maintaining Bmp signaling in cells and tissues (Brazil et al., 2015a). This fine regulation of Bmps is illustrated by genetic conditions where Bmp signaling has been altered (For review see-Kornak and Mundlos, 2003). For example mutations in BMPRIA cause juvenile polyposis syndrome (Cao et al., 2006), point mutations *ACVRI/Alk2* cause Fibrodysplasia ossificans (FOP), a genetic disorder resulting in heterotropic ossification (Shore et al., 2006). Autosomal dominant stapes ankylosis syndrome is attributed to mutations in Noggin (Brown et al., 2003). Many mouse mutants deficient in either Bmp or Bmp antagonists exhibit complex and often lethal phenotypes (Table 3.1). It is important to note that due to the

lethality caused by homozygous null Bmp and Bmp antagonist mutants, tissue specific deletion of Bmp/Bmp antagonists is often required when studying the role of Bmps.

Despite significant advances in deciphering modulation of Bmp signaling, many challenges remain. Many studies have been performed to understand the different molecular components of the Bmp pathway. More information is needed in order to understand how each component of the signaling pathway is integrated, and how cross talk of Bmps with other conserved signaling pathways such as Wnt, Hedgehog or Fgf signaling is controlling morphogenesis and tissue patterning during organogenesis.

We used tooth as a genetic model towards understanding the role of *Bmp2* in epithelial mesenchymal interaction during tooth development and how this forms minerilized structures of the tooth.

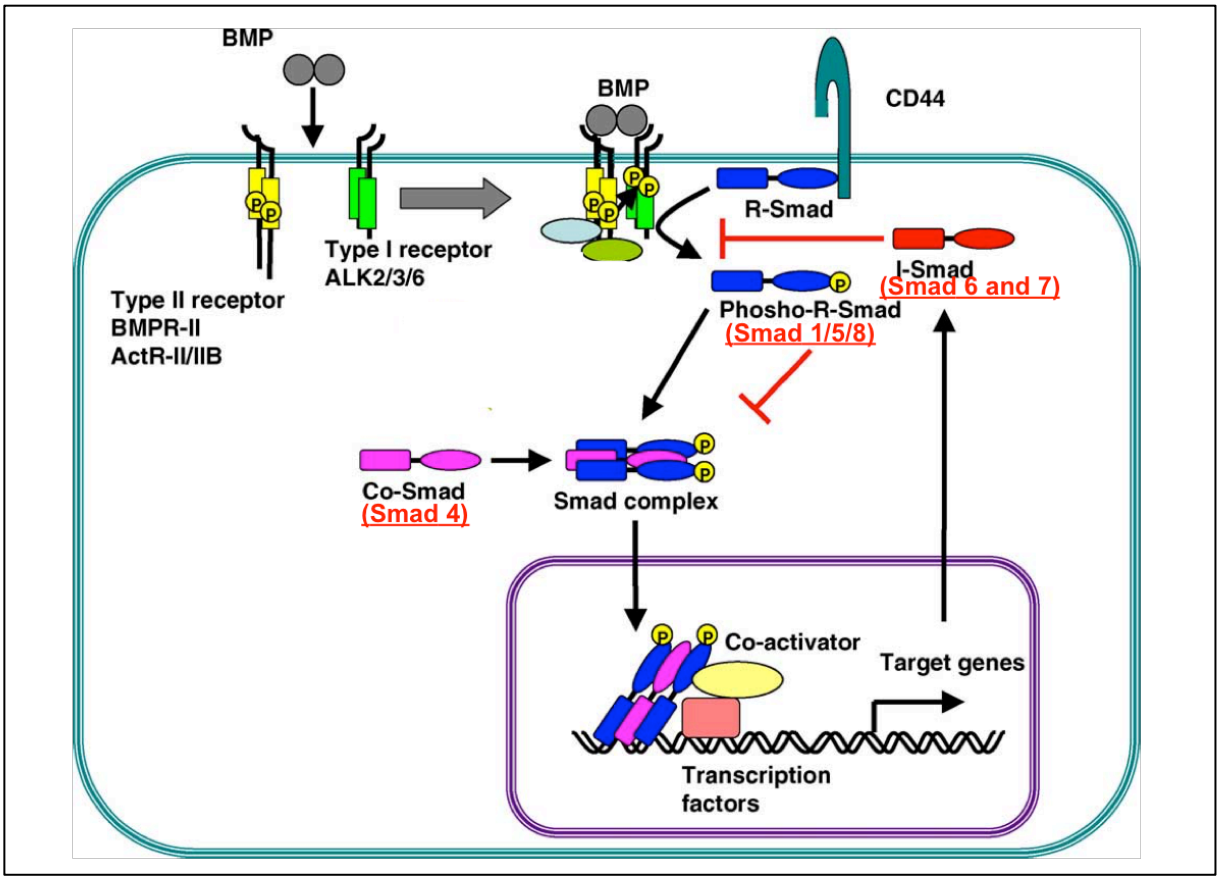


Fig 3.1 Schematic of Canonical Bmp Signaling. Bmps signal through two type I and type II receptors. Bmp ligands bind to type 1 receptor (BmprIs) or Activin like kinases (Alk), 2,3 or 6. This complex binds to type II receptors (BMPRII), phophorylating type I receptor in the GS glycine-serine domain. This is followed by phosphorylation of a set of Smads, known as receptor Smads (R-Smad1/5/8) that binds to nuclear Smad (Smad4). This complex is transported to the nucleus where it directs the transcription of the target gene (Modified from (Miyazono et al., 2005)).

Gene	Phenotype	Reference for null allele
Bmp2	Embryonic lethal: amnion defects, heart defects	(Zhang and Bradley, 1996)
Bmp4	Embryonic lethal: defects in mesoderm formation	(Winnier et al., 1995)
Bmp7	Perinatal lethal: kidney agenesis, eye defects, skeletal defects	(Zouvelou et al., 2009)
Noggin	Embryonic lethal: skeletal defects, CNS defects	(Brunet et al., 1998)
Gremlin1	Perinatal lethal: kidney, lung and skeletal defects	(Michos et al., 2004)
Chordin	Perinatal lethal: pharyngeal defects (DiGeorge syndrome)	(Bachiller et al., 2000)
Twisted Gastrulation	Variable penetrance depending on genetic background: embryonic lethal on C57Bl6 with craniofacial, skeletal, lymphoid, foregut defects	(Petryk et al., 2004)

Table 3.1- Phenotypes associated with members of Bmp family (from Graf and Economides 2009)

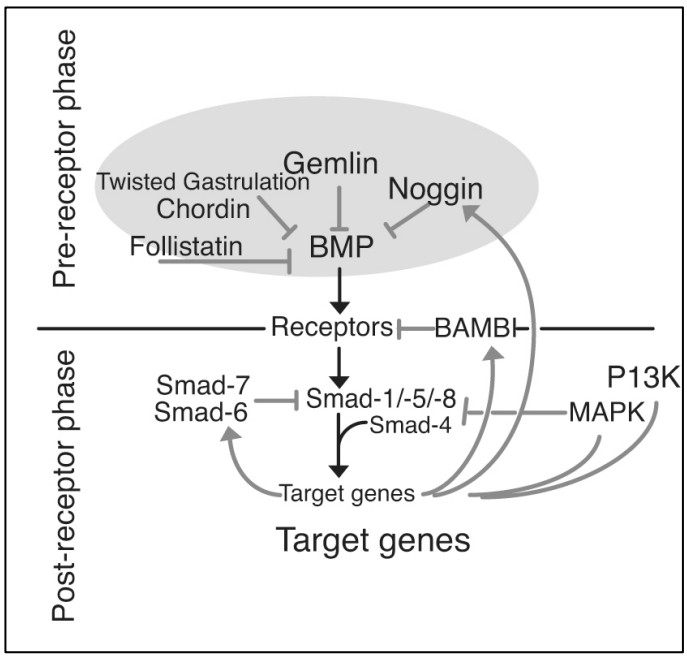


Figure 3.2: Negative regulation of Bmp signaling by Bmp antagonists (A) A balance exists between Bmp and Bmp antagonists, which controls the quantity and quality of Bmp signal transduced. Smad1/5/8 is a downstream Bmp event and is a read-out of Bmp signaling. MAPK and P13K are also wired to the Bmp signaling pathway but are not Bmp specific (from Graf and Economides, 2009)

3.2 Tooth: Structure and Function

The main function of teeth in humans is mastication. Every species has a unique set of teeth known as dentition. Emergence of a specialized dentition was an important event in evolution of the head. Because different species have different feeding habits, it is important that teeth maintain their unique shape and structure with proper anchoring to bone, to be able to carry out proper crushing and grasping of food. All vertebrate species have teeth or structures similar to teeth, known as denticles or odontodes, with few exceptions, where they are lost in some lineages (Jernvall and Thesleff, 2012). Teeth have been a source of immense information in fossils, as they contain enamel the hardest substance in the human body and thus are often well-preserved. There are two main theories about origin of teeth – the "outside-in" and "inside-out" theories. The "outside-in" theory proposes an inward progression of denticles. Denticles were found to be present on the body of many ancient jawless fish as protective covering. These covering were similar to the dentin like structure and can still be found in present-day sharks. This inward progression of the outer denticles formed the first primitive oral teeth in oral cavity. However the discovery of pharyngeal teeth in jawless vertebrates proposes a different view point on origin of teeth. This is referred to as "Inside-out" theory, which proposes an outward formation of pharyngeal teeth to form oral teeth (Tucker and Sharpe, 2004).

Many amphibians and most reptiles and fish are comprised of homodont (similar shape) dentition with large number of teeth (polyodont) undergoing continuous replacement (polyphyodont) (Huysseune and Witten, 2006). These teeth are composed of enamel, or enamel-like structure, dentin, with no roots, and are attached to the bone by ankylosis or fibrous tissue. In contrast, in mammals teeth are rooted in the alveolar bone and connected to the jaw by periodontal ligament (Figure 3.3). However, many vertebrates carry long-lasting or permanent dentitions, which requires a different type of anchoring to accommodate growth of the jaw and masticatory forces over the years (Jernvall and Thesleff, 2012).

Human teeth consist of two parts: crown and roots. The crown is composed of visible hard, inert, acellular enamel, underneath is the mineralized, yellowish white, avascular dentin (less mineralized than enamel but more protected towards outside stress), followed by the soft connective tissue known as pulp which houses nerves and putative odontoblast stem cells. Attachment of the tooth to the alveolar bone is by tooth supporting structures such as periodontal ligament, and cementum. (Nanci A, 2013).

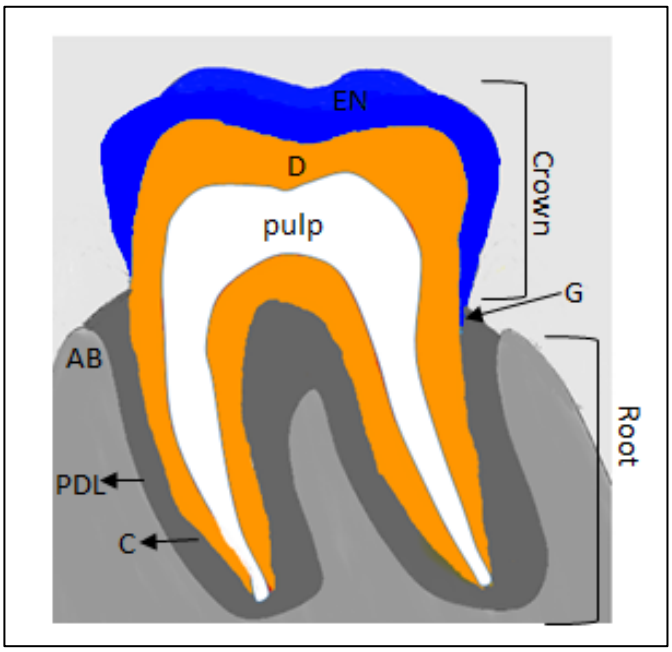


Figure 3.3 Depiction of the various structures of lower first molar of adult human. The tooth consists of two parts embedded in alveolar bone (AB)- Crown, with a visible enamel structure and root with tooth supporting structures such as periodontal ligament (PDL) and cementum (C). The four mineralized structures of the teeth are enamel (EN), dentin (D), periodontal ligament (PDL) and cementum (C). The pulp is the soft tissue of the tooth, which forms reservoir of the nerves and putative odontoblast stem cell. Gingiva (G) is the oral mucosa surrounding the tooth.

There are four different types of mammalian teeth: incisors, canines, premolars and molars. Irrespective of the type and shape of the tooth, development of all teeth follows a common principle. Studies on mutant mice have revealed various molecular pathways involved in tooth development (Jheon et al., 2013). Embryonic teeth can be grown in explant cultures for experimental manipulations, or transplanted to ectopic sites such as kidneys, where they grow into recognizable mineralized teeth (Tucker and Sharpe 2004).

Mouse is a common laboratory animal used to study tooth development. The mouse dentition consists of one set of teeth in each quadrant of the mouse jaw and is therefore considered to be a simplified model of human dentition (Line, 2003). Although the mouse dentition is attractive for genetic manipulation, there are important differences between human and mouse teeth. The mouse dentition consists of only two types of teeth: incisors and molars, in contrast to the human dentition, which consists of incisors, canines, premolars, and molars. Instead of canines and premolars the mouse jaw contains an edentulous space, often referred to as diastema (Figure 3.4). Molars of mice are structurally and developmentally similar to human molars. In contrast, incisors are quite different. Because mice are rodents and have significant wear on their incisors, their incisors grow continuously throughout their life and contain two distinct types of epithelia: lingual and labial epithelium. The lingual side is considered to be the root analogue, whereas the labial side is the crown analogue of molars. Another characteristic feature of mouse incisors is the presence of a cervical loop area (CL), where epithelial and mesenchymal stem cells reside. Cells from the CL area proliferate and migrate and form enamel producing cells called ameloblasts (Mitsiadis and Graf, 2009)(Figure 3.5).

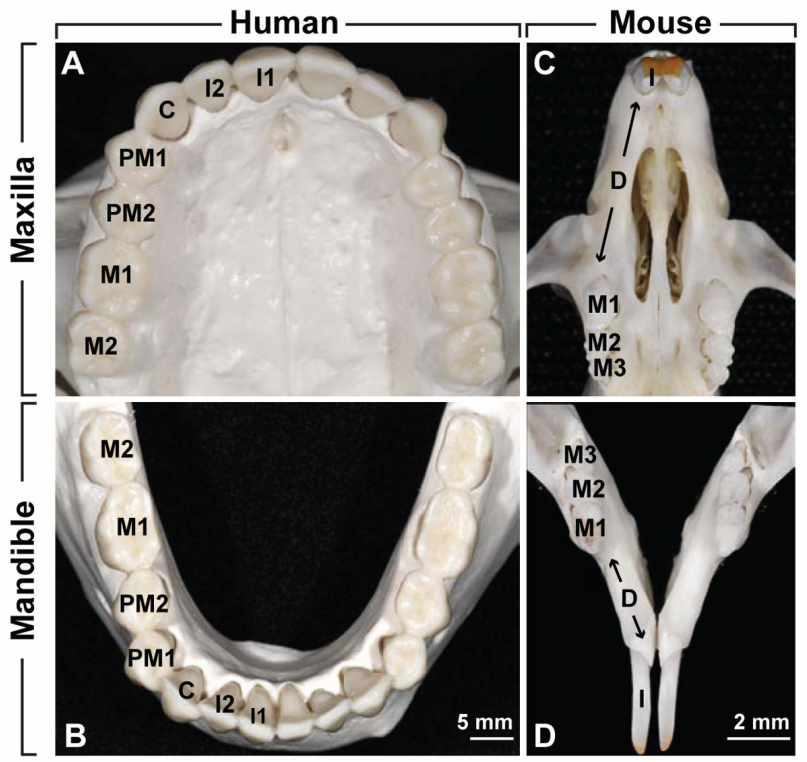


Figure 3.4 Comparison of Human and Mouse dentition. The maxillary (A, C) and mandibular (B, D) dental arches show the reduced dentitions in adult human (A, B) and mouse (C, D). Both species are derived from a common mammalian ancestor that is thought to have had 6 incisors, 2 canines, 8 premolars, and 6 molars in each dental arch. The third molar or wisdom tooth (M3) is absent in the human specimen. I, incisor; I1, central incisor; I2, lateral incisor; C, canine; PM1, first premolar; PM2, second premolar; M1, first molar, M2, second molar; M3, third molar; D, diastema. (Jheon et al., 2013)

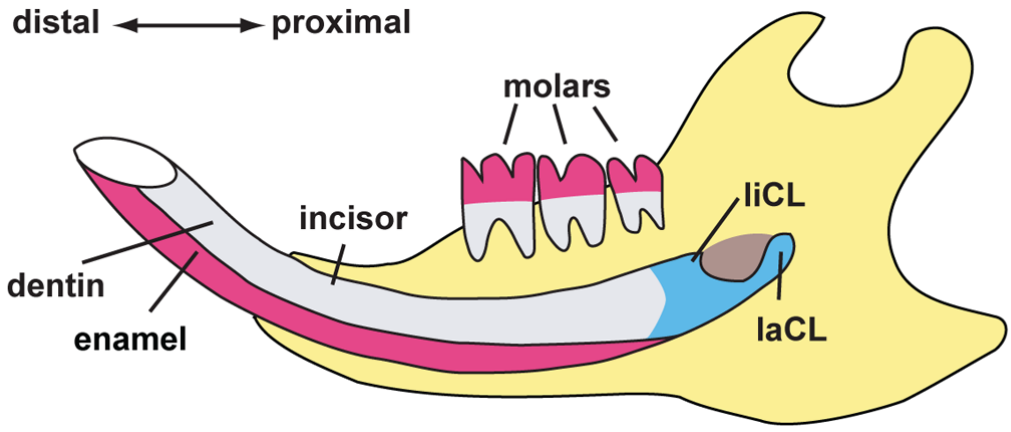


Figure 3.5 Adult mouse mandible (Line, 2003). Adult mandible showing molars and incisors. Dentin is shown in grey and enamel in pink. liCL-Lingual cervical loop; laCL-Labial cervical loop.

3.3 Tooth Development

Teeth like all epithelial appendages arise through reciprocal epithelial- mesenchymal interactions. In mammals the epithelium is derived from ectoderm and the mesenchyme from neural crest that migrates into first branchial arch and frontonasal processes. The initial sign of tooth development is formation of the dental lamina, formed along the mandible and maxilla as a horse-shoe shaped epithelial stripe (Jussila and Thesleff, 2012). Teeth are formed within the dental lamina. Following this, localized thickenings of the epithelial bands or placodes are formed within the dental lamina. Individual teeth are formed from the epithelial bud that invaginates the underlying mesenchyme giving rise to bud stage. The neural crest mesenchyme is specified as dental mesenchyme (Jernvall and Thesleff, 2000). Upon condensation of the mesenchyme the embryonic tooth acquires the cap shape, which contains the primary enamel knot, a structure thought to shape tooth. Lateral protrusions and extension of the epithelium and further condensation of the mesenchyme give rise to the bell stage with secondary enamel knots, which forms the crown of the tooth. At this stage, cytodifferentiation occurs and the mineralization front is initiated by enamel forming cells called ameloblasts and dentin forming cells, odontoblasts (Tucker and Sharpe, 2004). It has been shown that ameloblasts require signals from functional odontoblasts for their differentiation (Zeichner-David et al., 1995). *In vitro* studies have shown that signals from odontoblasts induce ameloblasts, but if this is required *in vivo* is not known (Coin et al., 1999).

Root formation with tooth supporting structures such as periodontal ligament and cementum are formed during late stages of tooth formation, usually after end of the first postnatal week in mouse (Huang and Chai, 2012) (Figure 3.6). Each of the specialized dental hard tissues along with developmental anomalies is described in the following sections.

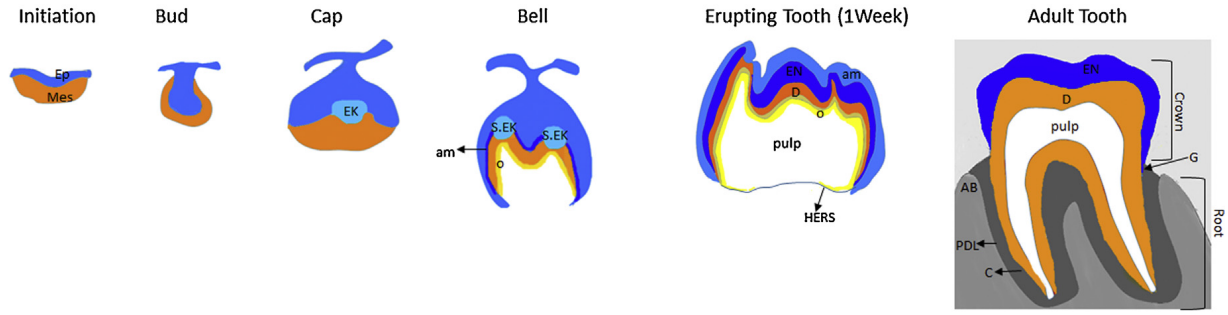


Fig. 3.6 BMP signaling in tooth. Schematic representation of tooth morphogenesis and of an adult tooth. Ep-epithelium, Mes-mesenchyme, EK-enamel knot, SEK-secondary enamel knot, o-odontoblasts, EN-enamel, d-dentin, Am-ameloblast, AB-alveolar Bone, PDL-periodontal ligament, C-cementum, G-gingiva. (Graf et al., 2016)

3.4 Enamel formation (Amelogenesis)

Enamel is an epithelial derived structure covering the crown of the tooth. It consists of 96% mineral and 4% organic material and water. Inorganic components of enamel include crystalline calcium phosphate (hydroxyapatite). The high mineral content of enamel makes it the hardest structure in body (Gibson et al., 2001). This property along with complex structural organization allows it to withstand the mastication forces, thereby fulfilling the main function of the tooth. Hardness of enamel makes it brittle, therefore the underlying more resilient dentin layer is important for maintaining the integrity of enamel. The fundamental organizational units of enamel are rods and interrods (Nanci A 2013) (Figure 3.7).

Enamel formation begins at crown forming stage of development. This involves differentiation of inner enamel epithelium at the tips of the cusps, continuing down to the slope of the tooth crown until all the cells of epithelium have differentiated into ameloblasts (enamel forming cells) (Bartlett, 2013). Ameloblasts secrete matrix proteins and maintain favorable environment for mineral deposition. There are various steps and stages through which ameloblasts take part in enamel formation. These stages can be divided in as many as seven stages (Figure 3.8), but the main functional stages are divided into three phases-presecretory, secretory and maturation phase. During the presecretory stage ameloblasts

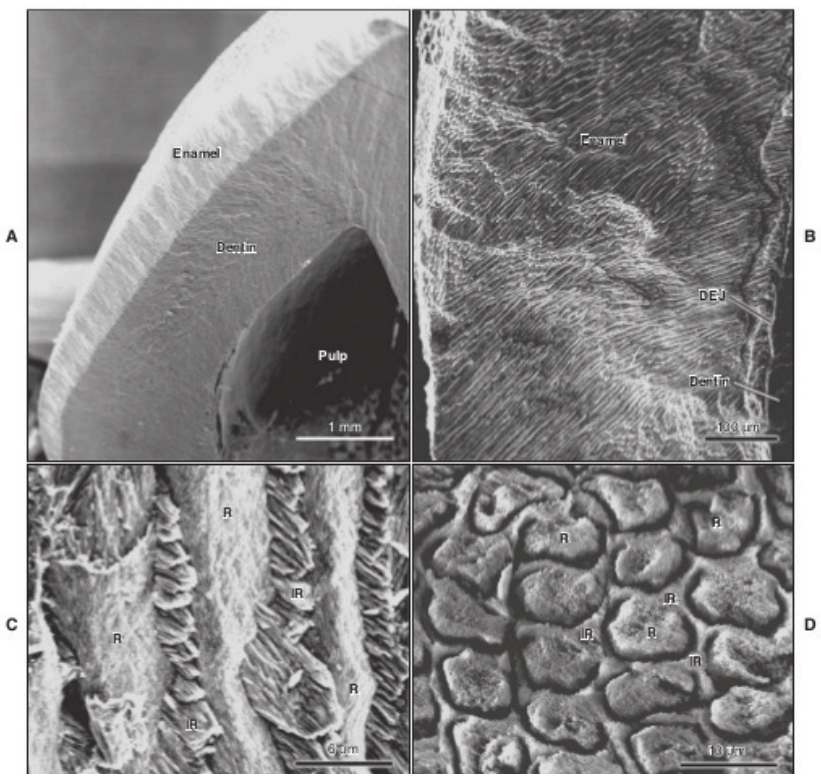


Figure 3.7 Enamel structure. Scanning electron microscopy of (A) Enamel layer covering dentin (B) Enamel rod distribution (D) Rod, interred relationship (C) longitudinally and (D) in cross section. (from Nanci A 2008)

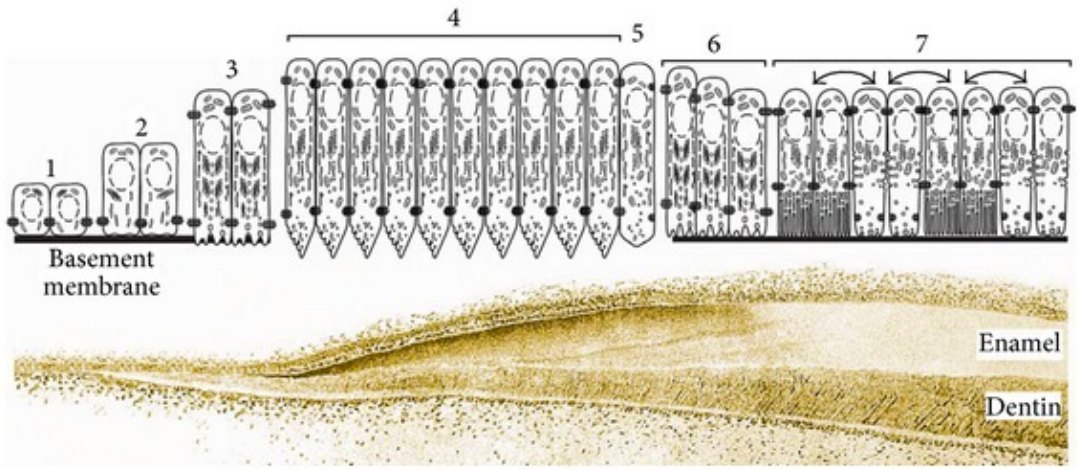


Figure 3.8 Ameloblast structure during different stages of enamel formation. (1) Differentiation of ameloblasts starts (2) Presecretory ameloblasts (3) Secretory ameloblasts develops a secretory specialization or Tomes' process. Enamel crystals grow in length as secretory ameloblasts come into play (4). Each enamel rod follows a retreating Tomes' process from a single ameloblast. (5). At this point, the enamel has achieved its final thickness. Secretory activity of ameloblasts diminishes and ameloblasts undergo restructuring with changes in types of proteins secreted (6). KLK4 is secreted, which degrades the accumulated protein matrix. During the maturation stage ameloblasts modulate between ruffled and smooth-ended phases (7). Their activities harden the enamel layer. (from Barlett J 2013).

develop an extensive protein synthesis machinery, change polarity and prepare themselves to secrete organic matrix. During the secretory stage ameloblasts become tall, elongated columnar cells and form Tomes processes at the apical end. It is from the secretory side of the Tome`s processes that enamel matrix proteins are secreted and form crystallite ribbons that lengthen. The maturation stage is marked when enamel has reached its full thickness and ameloblasts transition into small protein reabsorbing cells allowing enamel to reach its final hardened form (Hu et al., 2008). Ameloblasts secrete at least four different enamel matrix proteins at the secretory stage: amelogenin (Amlx), ameloblastin (Ambl) and enamelin (Enam) and metalloproteinase-20 (MMP20) (Papagerakis et al., 2008). Mutations in any of these genes have been associated with amelogenesis imperfecta (AI), a clinical condition where disruption of the formation of enamel or clinical appearance of enamel is distorted (Crawford et al., 2007).

3.5 Dentin formation (Dentinogenesis)

Dentin is a mesenchyme-derived structure, initially deposited as an unmineralized layer called predentin (10-50mm thickness) forming the innermost portion of the dentin, also referred to as pulpal dentin portion. Predentin principally consists of collagen and is easy to distinguish from dentin as it stains less intensely in histological stains (Figure 3.9) (Nanci A 2013). As various non-collagenous matrix proteins start to be incorporated at the mineralization front, the predentin gradually mineralizes into dentin. Mature dentin is made up of approximately 70% inorganic material, 20% organic material and 10% water (Arana-Chavez and Massa, 2004)(Verdelis et al., 2007). The inorganic component consists of substituted hydroxyapatite in form of small plates. The organic phase consists of mostly collagen with fractions of various non-collagenous proteins. There are three types of dentin: primary dentin, secondary dentin and tertiary dentin (Cao et al., 2015). Most of the dentin is made up by primary dentin. Dentin formation begins at late bell stage of development by mesenchymal derived cells called odontoblasts. The initial or primary dentin forms the crown

dentin (Goldberg et al., 2011). The secondary dentin forms later in life, whereas tertiary dentin forms in response to injuries (reparative dentin). Root dentin is formed at later stages of tooth development and requires interaction with epithelial cells, HERS (Hertwig`s epithelial root sheath), a derivative of the enamel organ (Zeichner-David et al., 2003) (described in next section).

Differentiation of odontoblasts initiates dentin formation. For this, odontoblast precursor cells migrate from the pulp while undergoing a fixed number of cell divisions to reach the dentin-pulp border. Differentiation of odontoblasts is characterized by increased size and highly polarized structure with their nuclei positioned away from inner enamel epithelium. Structurally, odontoblasts align to the surface of dental pulp with a palisade structure (Arana-Chavez and Massa, 2004). After the differentiation of odontoblasts, formation of the organic dentin matrix is initiated, marked by the appearance of collagen fibrils (0.1-0.2mm diameter). Along with the deposition of the collagen, odontoblasts also bud off small membrane vesicles known as matrix vesicles. The mineral phase forms in the matrix vesicles crystals, which rupture from the vesicles to form continuous mineralized matrix layer (Butler, 1998). Odontoblasts can be morphologically subdivided into various stages ranging from the active synthetic phase to the quiescent phase (Figure 3.10). Non-collagenous proteins (NCPs) secreted by odontoblasts regulate the mineral deposition following the mineral seeding. They include Dentin phosphoprotein (Dpp), Dentin Sialoprotein (Dsp), Dentin glycoprotein (Dgp), Dentin matrix protein (Dmp1), osteonectin, osteocalcin, bone sialoprotein (Bsp), proteoglycans and some serum proteins (Butler, 1998)(Fisher and Fedarko, 2003). Dpp, Dsp and Dgp are all expressed by the Dspp (Dentin sialophosphoprotein) gene, which is processed into each component depending on their properties (Napierala et al., 2012). DSPP mutations have been shown to be associated with dental anomalies, which include dentin dysplasia and dentinogenesis imperfecta affecting both primary and permanent dentition (Kantaputra, 2001). There are three types of dentinogenesis imperfecta (DI): type 1 is associated with osteogenesis imperfecta caused by mutations in collagen type I. In DI type 1 and 2 the pulp chamber is very small due to abnormal dentin deposition. Mice

deficient in *Dspp* or *Dmp1* show enlarged pulp, thickened predentin and hypominerilization (MacDougall et al., 2006). Though there is a wealth of data available on various phenotypic features in mutant mice with regards to dentin formation, more information is needed to understand the molecular mechanisms regulating tooth hard tissue formation.

Figure 3.9 Cross section of a mouse incisor showing different layers of tooth. Note distinctive H&E stain of predentin (pd), dentin (d) and enamel (e). Od-odontoblasts, ame-ameloblasts

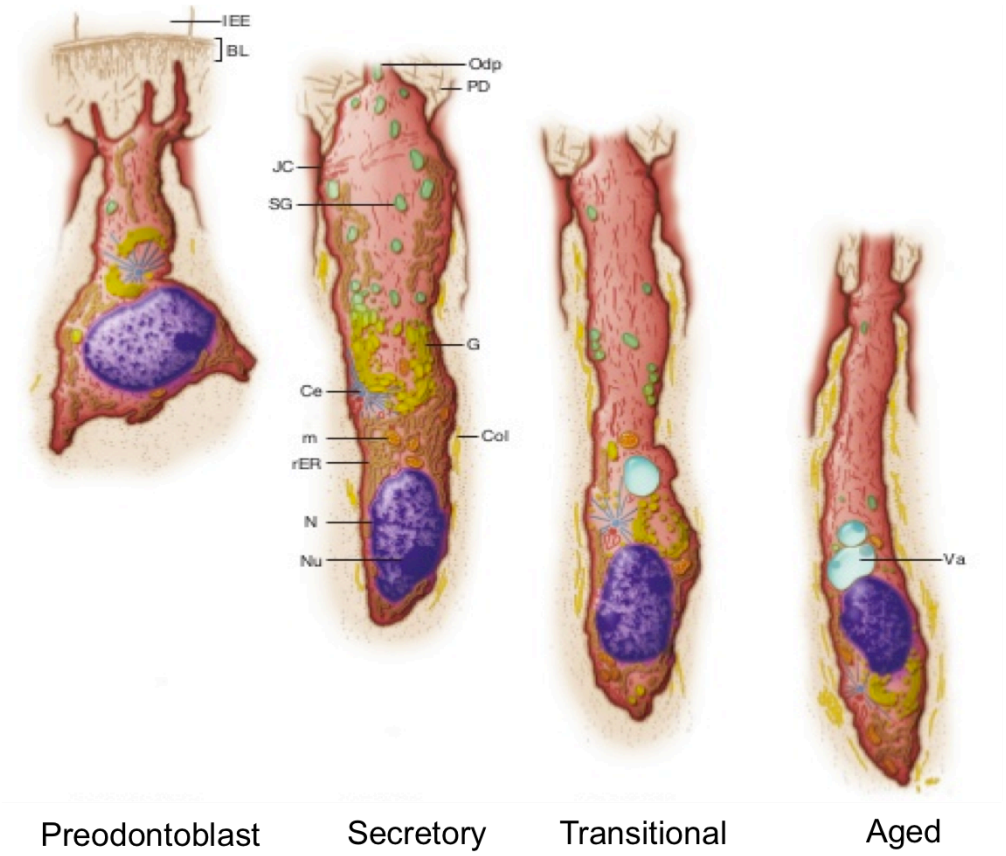


Figure 3.10 Schematic of functional stages of odontoblasts. BL-Basal Lamina, Ce-centriole, Col-Collagen, G-Golgi complex, IEE-Inner enamel epithelium, JC-Junctional complex, m-mitochondria, N-nucleus, Nu-Nucleolus, Odp-Odontoblast process,PD-predentin,rER-Rough endoplasmic reticulum, SG-Secretory granule, Va-Vacuole (Adapted from EArch Oral Biol 31:463, 1986 and Nanci 2013)

3.6 Root formation

Tooth root development begins after crown formation is near to completion. An important layer of epithelial sheath called HERS guides root formation. Epithelial root sheath is located between dental follicle and dental papilla- structures derived from dental mesenchyme (Figure 3.11). HERS are formed from inner and outer enamel epithelium at the neck of the crown and proceeds in an apical direction. As HERS grow in apical direction the dental papilla cells adjacent to inner enamel epithelium induce formation of odontoblasts and during later stages to form root dentin. After the formation of root dentin HERS become perforated and interrupted. These HERS facilitates the newly formed root dentin to contact with dental follicle cells and allows differentiation of cementoblasts, which in turn form cementum. Also there are reports suggesting that some HERS undergo epithelial to mesenchymal transition

to form cementoblasts, which gives rise to cementum. The collagen fibers from the dental follicle help in fixing the root in the bone of the jaw. After tooth root development tooth erupts (Bosshardt et al., 2015; X. F. Huang et al., 2009; Huang and Chai, 2012; Xu et al., 2009). During the terminal stages of root development it is not clear how HERS move away from root surface. Studies have shown that HERS migrate away from root surface and form epithelial rests of Malassez, but the mechanisms, which are involved in this, are not clear yet (Figure 3.11) (Luan et al., 2006).

HERS are different than epithelial cells of the crown, as they do not respond to signals from the mesenchyme to differentiate into ameloblasts. The main function of HERS is differentiation of cementoblasts and odontoblasts, root growth, and also root number (Xu et al., 2009). Any disruption in epithelial root sheath formation/HERS results in root defects (X. Huang et al., 2009). In summary, HERS may be considered as developmental center for root formation.

Disorders of root development in humans are briefly categorized in the following groups- Premature arrest of tooth development, root dilacerations, root defects associated with cervical mineralized diaphragm, short root anomaly and taurodontism (Luder, 2015). A detailed review on various human root disorders is summarized by Luder H, 2015.

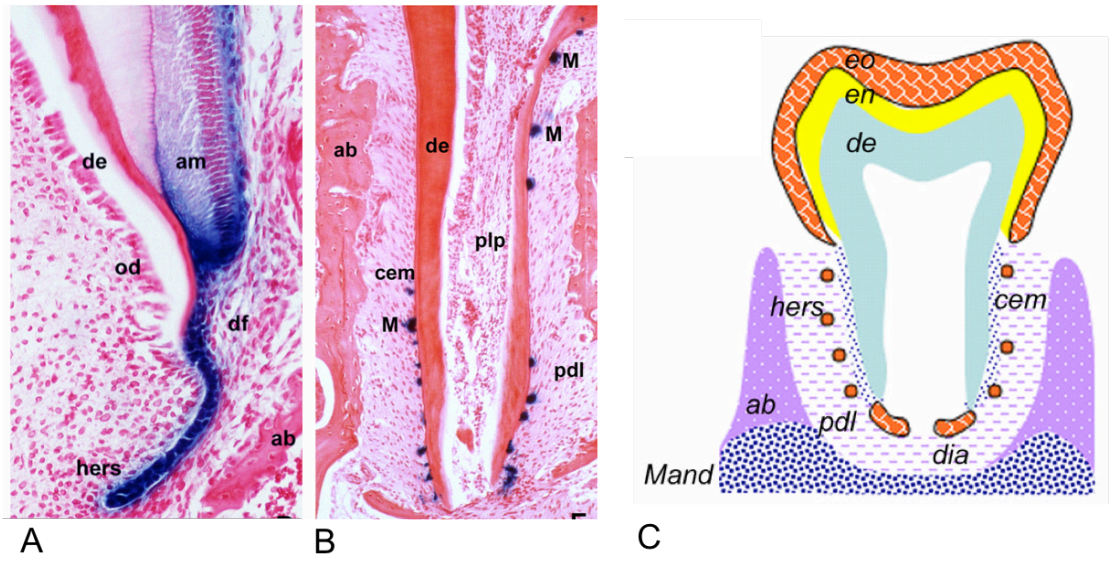


**Figure 3.11 Hertwig`s Epithelial Root Sheath (HERS)**. (A) Initial stage of root formation where the Hertwig`s epithelial sheath is continuous in lower molar of one week old postnatal mice carrying the K14 transgene. Ameloblasts (am) and HERS are stained. (B) Section through lower molar of six weeks old postnatal mice. At stage HERS are reduced to Epithelial cell rests of malassez (M) which are positive for K14 transgene. (C) Schematic of the cross section of a mammalian tooth during root development. Periodontal ligament (pdl) is well organized into the fiber bundles along the root. HERS stretches along the entire surface of the root, alveolar bone (ab) on the side provides anchorage to the root. Abbreviations- de-dentin,od-odontoblasts,am-ameloblasts,df-dentalfollicle,ab-alveolar bone,cem-cementum,dia-diaghragm, eo-enamel organ,en-enamel Modified from (X. Huang et al., 2009)

3.7 Cementum and Periodontal Ligament

Cementum covers the tooth root surface of a mature mammalian tooth. The stability of the structure is provided by periodontal ligament, which is embedded in both cementum and alveolar bone. There are two types of cementum – acellular and cellular cementum. Cellular cementum forms one-third of the apical root and acellular cementum covers the rest. Cellular intrinsic fibre cementum (CIFC) has also been found in some teeth upon root injury. The contribution of the epithelial and mesenchymal components in cementogenesis remains a subject of debate but previous studies have suggested that these are dental follicle derived connective tissues (Diekwisch, 2001).

Periodontal Ligament is derived from the dental follicle. Formation of periodontal ligament begins when HERS dissociation is detectable. The short collagen fibers attach to the root

surface and grow into the periodontal space. Fibers embedded in cementum are called Sharpey`s fibres. They are formed by fibroblasts and stem cells in the dental follicle and grow thick and organized specialized periodontal fibers, which help in the attachment of tooth to the alveolar bone and gives rise to a fully functional tooth (Palmer and Pritlove-Carson, 1992) (Huang and Chai, 2012).

3.8 Signaling networks in developing tooth and molecular regulation mechanism of dental hard tissues

All stages of tooth development are regulated by epithelial-mesenchymal interactions, involving four main conserved signaling pathways: Fgf (Fibroblast growth factor), Wnt, Hh (Hedgehog) and Bmp (Bone morphogenetic proteins) (Thesleff and Sharpe, 1997). An overview of general signaling involved in tooth development is described in this section with a more detailed overview on Bmp signaling in tooth in the following section.

There are three epithelial signaling centers formed during the course of development: placode, primary enamel knot and secondary enamel knot. All ectodermal organs share similar molecular mechanisms during placode formation (Mikkola, 2009). An important placode development gene is *p63.* Studies in mice have shown that deletion of *p63* results in tooth arrest before epithelial budding (Bei, 2009). An array of signaling pathways, for eg. Bmp, Notch, Fgf, Ectodysplasin, is disrupted in *p63* mutant mice (Laurikkala et al., 2006). Another important molecule important in the development of placode is Eda (Ectodysplasin). In humans, mutations in EDA pathway genes have resulted in HED (Hypohdrotic Ectodermal dysplasia), characterized by missing teeth, sparse hair and reduced sweating (Mikkola 2009b). Mutant mice overexpressing Edar have shown to develop supernumerary teeth, hair and sweat glands (Mustonen et al., 2003). The target molecules of Eda signaling include Shh, Fgf20, Dkk4, Ctgf and follistatin. Thus Eda signaling may be considered as a key regulator of ectodermal organ development (Mikkola 2009b). Many members of the Wnt family have also been shown to be expressed in early dental epithelium (Wang et al., 2009). Mutation in the Wnt inhibitor Dkk1 in mice arrests placode formation (Andl et al., 2002). A

recent study by (Bloomquist et al., 2015) in mouse and fish have shown that Wnt–Bmp-Hh regulatory hierarchy in taste buds and teeth organs during placode development is conserved. Classic experiments performed on recombination of tissues have shown that odontogenic potential shift from epithelium to mesenchyme at around E11 and E12 in mouse. This shift is marked by expression of various transcription factors. Targets of Bmp and Fgf signaling in mesenchyme include Dlx1/2, Barx1, Runx2, Lhx6,7 (Bei, 2009). Expression of the important Wnt molecule Lef1 shifts from epithelium to mesenchyme along with the shift in odontogenic potential (Kratochwil et al., 1996).

During bud to cap morphogenesis primary enamel knot appears in the dental epithelium. It directs crown formation and also determines the position of secondary enamel knot. Requirement of Wnts in enamel knot formation is shown by induction of new enamel knots upon forced activation of Wnt/β-catenin signaling in oral epithelium (Wang et al., 2009) (Järvinen et al., 2006). Wnt signals are also important in bud to cap stage transition (J. Chen et al., 2009). A lot of different signaling molecules are expressed in enamel knots from all four conserved signal families. The enamel knots regulate the folding of the epithelium by proliferation through Fgfs (Fgf 3,4,9,20). However enamel knots do not proliferate themselves. They express p21 (cyclin dependent kinase inhibitor) making them non-proliferative (Jernvall et al., 1998). An important role attributed to the enamel knot is formation of the cusp shape and cusp patterning in molars. Several studies have provided experimental support to prove this hypothesis. For example *K14 Eda* mutants exhibit teeth resembling kangaroo teeth. *Sostdc* (inhibitor of Wnt and Bmp signaling) mutant resembles rhino teeth (Kangas et al., 2004; Kassai et al., 2005). Also epithelial deletion of *Dicer* (required for miRNA processing) modulates molar cusp patterning (Michon et al., 2010).

Formation of supernumerary teeth in mutant mice has been observed for the following mutants: *K14-Eda* mouse (Mustonen et al., 2003), *Sprouty* mutants (Klein et al., 2006) and *Osr-2* knockout (Zhang et al., 2009).

During the bell stage of tooth development *Wnt10b* has been suggested to regulate *Dspp* expression, and in turn odontoblast differentiation (Yamashiro et al 2007). For ameloblast

differentiation *Shh* has been shown to be an important signal from the epithelium of stratum intermedium (Dassule et al., 2000; Gritli-Linde et al., 2002). Growth factors involved in ameloblast differentiation are Wnt3, Eda, Follistatin, Tgfβ1 (Bei 2009a). Transcription factors expressed by ameloblasts in mice are *Msx2* and *Sp6* (Bei 2009a).

Various genes are expressed during root formation (Huang and Chai, 2012). Tgfβ signaling has been shown to be important for root dentin and cementoblast formation (Oka et al., 2007). Deletion of *Smad4* (central mediator of canonical Tgf-β as well as Bmp signalling) in dental mesenchyme shows short root anomaly and defects in dentin formation. It also up regulates Wnt signaling and downregulates *Dkk1* and *Sfrp1* (Wnt inhibitors). *In vitro* studies have shown that exogenous Tgfβ1 can induce odontoblast differentiation in dental papilla cells (Smith et al., 1998). *Smad4* deletion in epithelium results in arrested molar root development. It was shown that Smad4 mediated Tgfβ/Bmp signaling is required for *Shh* expression in HERS and *Nfic* expression in dental mesenchyme (Huang et al., 2010). Nfic is a downstream target of Tgf-β1 signaling during tooth root development (Lee et al., 2009). Mutant mice deficient in *Nfic* show normal crown development but root development is defective with abnormal dentin formation (Park et al., 2007).

3.9 Bmp signaling in tooth development

Bmps are expressed during all stages of tooth development. Fine-tuning of Bmp signals is important from the early stages of tooth development. Bmp signals are important in mouse incisor placode formation, which in turn determines the number of incisors that will develop (Munne et al 2010). Continuous growth of enamel formation in mouse incisors is modulated by Fgfs, Activin and Bmp signaling in epithelial stem cell niche (Wang et al 2007). Early signals in the jaw epithelium include that of Bmp4 and Fgf8 in overlapping patterns before tooth germ undergoes any morphogenesis (Neubüser et al., 1997). Bmp4 expression in the mesenchyme is induced by epithelial Bmps marking a shift in odontogenic potential from epithelium to mesenchyme at E12 in mouse (Vainio et al., 1993). Also it has been shown

that Bmp4 is stimulated by Wnt/β-catenin signaling in the incisor mesenchyme, which in turn regulates Shh in epithelium (Fujimori et al., 2010). *Bmp4* is an important signal involved in bud to cap transition, besides Wnt molecules, as mentioned in the previous section. Bmp4 deletion in dental mesenchyme results in arrest of mandibular molars at the bud stage, with altered expression of Pax9 (Neubüser et al., 1997) and Msx1 (Chen et al., 1996; Vainio et al., 1993). *In vitro* experiments have established that Bmp4 regulates the expression of Msx1 and Pax9. In humans, MSX1 or PAX9 mutations are associated with selective tooth agenesis (Vastardis et al., 1996). Bud stage arrest has also been observed in Bmpr1a mutants (Andl et al., 2004) indicating importance of mesenchyme signals in tooth epithelium. Overexpression of Noggin results in loss of mandibular molars in mice. Various Bmps are expressed in primary enamel knot, but their precise role has not been directly assessed (Jussila and Thesleff, 2012). Supernumerary tooth formation by Ectodin/Usag-1 has shown to be regulated by Bmp2 and Bmp7 in cultured tooth (Laurikkala et al., 2003).

Bmps are expressed during the differentiation stages of ameloblasts and odontoblasts. Bmp2, Bmp4 and Bmp7 have been associated with induction of ameloblastin expression, a protein associated with enamel formation. Follistatin, a Bmp antagonist has been shown to regulate enamel formation in mouse incisors controlling lingual – labial patterning. *Bmp2* deletion in odontoblasts and ameloblasts is associated with deregulation of enamel formation, with deregulated expression of enamel forming genes, such as *Amel*, *Klk4*, *Enam*, *Mmp20*. In humans mutation in any of these genes is associated with amelogenesis imperfecta (Hart et al., 2004)(Kim et al., 2005)(Mårdh et al., 2002). Bmp2 also regulates odontoblast differentiation and dentin formation. Although there is no molecular mechanism available to explain how mesenchyme derived *Bmp2* is important in enamel formation, the studies mentioned above suggests E-M interaction during the early stages of mineralization of teeth.

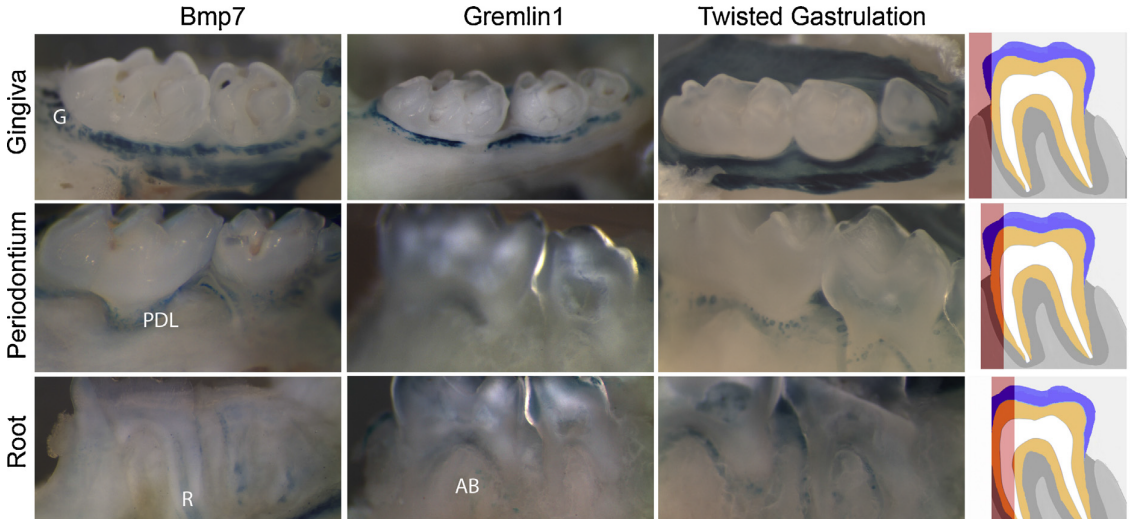


Fig. 3.12 BMP signaling in adult teeth. Whole mount lacZ stained molar teeth from 4 to 6 month old mice. Scheme on the right hand side indicates visible area (red) when viewed from the left. Gingiva = unmanipulated tooth, periodontium = gingival tissue resected, root = periodontium and tooth partly mechanically grounded off (Graf et al., 2016)

	Mice	Defects	References
Follistatin	*K-14* transgenic	Enamel patterning and ameloblast differentiation	
Bmp2	*SP7*Cre-Conditional KO *Osx*Cre-Conditional KO *Col1a1*-Cre Conditional KO	Root development and periodontium Enamel development and formation Odontoblast differentiation, delay in amelogenesis	 (Guo et al., 2015) (Yang et al., 2013)
Bmp7	Null	Various craniofacial deformities with missing maxillary molars and defective mandible.	(Zouvelou et al., 2009)
Bmp4	*Wnt1*-Cre Conditional KO	Tooth arrest at bud stage	(Jia et al., 2013)
Noggin	*K-14*- Cre transgene KO *Nog-pMes K14* (*Nog* overexpression mouse model)	Root/crown patterning defects Arrested tooth development at early bud stage.	(Plikus et al., 2005) (Yuan et al., 2015)
Bmpr1a	Conditional KO in epithelia at E14.5 *K14*- Cre	Cementoblast like cells in crown of epithelia. Defects in differentiation of crown epithelia Tooth arrest at bud stage	(Yang et al., 2013) (Andl et al., 2004)
Ectodin	Null	Supernumerary teeth	(Murashima-Suginami et al., 2008)
Grem2	Null	Incisors most severely affected with dentin and enamel defects.	(Vogel et al., 2015)

Table 3.2 Various Bmp mouse mutants with defects in tooth development.

3.10 Bmp signaling in root formation and adult tooth

Bmp2, Bmp3, Bmp4, Bmp7 are all expressed in the tooth root (Yamashiro et al., 2003). *Bmp2* ablation in mesenchyme shows defects in periodontal ligament and root development (Rakian et al., 2013). Overexpression of Noggin results in various defects including disrupted root development (Plikus et al., 2005). GREM2 mutations in humans have shown to be associated with taurodontism, microdontia, and short root. However Grem2 null mice only show defects in mandibular and maxillary incisor, and no root defect has been reported (Vogel et al., 2015).

There is relatively little information available during later mineralization stages and adult root formation as compared to the early tooth development stages. Bmp signaling has been associated with orthodontic tooth movement (Xu et al., 2013)(Takimoto et al., 2015). As shown in Figure 3.12 various Bmps operate in adult tissues (mouse 4-6 months old) indicating life-long requirement of Bmps in adult tooth in homeostasis and repair. In recent years more information has also come through various Bmp mouse mutants (Table 3.2) however there is more information needed in order to understand the molecular mechanisms regulating dental hard tissue formation and maintenance of tissue homeostasis in adult tooth.

3.11 Bmp2 in Tooth

In this particular study we analysed the role of *Bmp2*. Bmp2 has ben extensively studied for its role in chondrogenic and osteogenic differentiation and postnatal skeletal formation (Singh et al., 2008; Zhang and Bradley, 1996)(Tsuji et al., 2010, 2008, 2006). In tooth Bmp2 has been shown to be expressed in both odontoblasts and ameloblasts from cytodifferentiation stages onwards (Aberg et al., 1997). Various reports have shown the ability of Bmp2 to induce ameloblasts and odontoblasts *in vitro* making it an interesting candidate to study its *in vivo* role (Chen et al., 2008; Miyoshi et al., 2008). Also, Bmp2 is an important factor to differentiate dental follicle cells into cementoblast/osteoblast cell fate *in vitro*(Zhao et al., 2002*)*. More recent reports have shown an *in vivo* role of *Bmp2*. Conditional

deletion of *Bmp2* in epithelium is associated with amelogenesis imperfecta like phenotype (Guo et al., 2015) and conditional deletion of *Bmp2* in mesenchyme results in root and periodontium phenotype (Rakian et al., 2013). However the molecular mechanisms how *Bmp2* plays a role during development to confer these effects is still not known. Here we show *Bmp2* requirement in both epithelial and mesenchyme derived structures of the tooth with a focus on molecular dissection of its role and its possible role in formation of adult hard tissue structures of the tooth.

4. Aims of the Study

The aim of the project was to study Bmp signaling in epithelial-mesenchymal interaction during tooth organogenesis. Specific aims are as follows:

1. To address the role of *Bmp2* in tooth epithelium- We used K-14 Cre to conditionally delete *Bmp2* in epithelial derivatives of the tooth.

2. To address the role of *Bmp2* in tooth mesenchyme- We used Wnt1- Cre to conditionally delete *Bmp2* in mesenchyme (neural crest) derivatives of the tooth.

3. To address *Bmp2* function in adult tooth homeostasis- For this embryonic tooth germs were transplanted on kidneys of the recipient mice for in vivo ectopic growth of *Bmp2*-deficient teeth as *Bmp2* fl/fl: Wnt1-Cre mice die at birth.

5. Material and Methods

5.1 Animals

All animal experiments and animal housing was performed in accordance to the Swiss Animal Welfare Law and were in compliance with Cantonal Veterinary Office, Zurich and according to HSLAS guidelines at University of Alberta under license (AUP no. 1149). Mice deficient in *Bmp2* in mesenchymal cells were generated by crossing *Bmp2* floxed (*Bmp2* fl/fl*)* (Graf and Economides 2009) with a Wnt1-Cre (Chai et al., 2000) allele *(Bmp2* fl/wt: Wnt1-Cre*).* Mice deficient in *Bmp2* in epithelium were generated by crossing *Bmp2* floxed/ko (*Bmp2* fl/ko*)* with a K14-Cre (Hafner 2004) *(Bmp2* fl/wt: K14-Cre). For timed matings adult mice were mated overnight and the day of vaginal plug was designated as day 0.5. Recipient mice for kidney capsule transplantation were *Bmp2* fl/fl mice. Lineage tracing of K14-Cre was performed by crossing K14-Cre mice with Rosa26/LacZ (Soriano, 1999) reporter mice.

5.2 LacZ staining

Embryos, postnatal head, and postnatal mandibles were carefully dissected in cold PBS and jaw was opened (for postnatal head and embryos). They were fixed in fixative solution for 10 minutes (fixative buffer with 1:20 dilution of 37% formaldehyde and 1:80 dilution of 100% gluteraldehyde solution). This was washed in 1XPBS with 2mm $MgCl_2$ twice for 10 minutes each. X-gal staining was performed overnight at room temperature by dissolving X-gal stain in X-gal solution (1:50 dilution). After desired staining was attained samples were post fixed in 4% PFA overnight, washed with 1XPBS and processed for paraffin embedding. LacZ staining of adult tissues was done by perfusing anaesthetized mice with 10ml PBS followed by 10ml permebealization solution and 10ml lacZ solution. Desired tissues were dissected, and placed in staining solution for several hours under constant movement till desired staining was achieved. To stain the pulp chamber, teeth were physically opened using a file.

5.3 Micro- computed tomography (µCT) analysis

µCT analysis was performed using a µCT unit (Specimen µCT 40, Scanco Medical, Brüttisellen, Switzerland) voltage 70 kV, tube current 114 µA; and some micro-CT scans were performed at Prof Hallgrimson Lab at University of Calgary, Canada. The scanned images were converted into the RAW-format using the software of the micro-CT device and were analysed using a 3D reconstruction program VGStudio Max (Volume Graphics, Heidelberg, Germany) and Amira 3D software (FEI) at UofA (for volumetric analysis). The analysed samples were segmented manually using a wild type tooth as reference for the grey level values corresponding to the single mineralized tissues (enamel, dentin, bone).

5.4 Kidney Capsule Transplantation

Embryonic teeth or tooth germs were dissected from E13.5- E14.5 mouse embryos and were put in culture overnight at 37°C, 5%CO_2. Genotyping was performed and the mutant (*Bmp2 fl*/fl; Wnt1-Cre) and control (*Bmp2* fl/fl) teeth were transplanted under the peritoneum of the kidney of a *Bmp2* fl/fl mice.

The mice were 2-3 months old, anaesthetized using isoflorane inhalation. The kidney was exposed using micro surgical scissors. An incision was made on outer membrane of the kidney and samples were placed underneath the kidney membrane (see Figure 5.1). The kidney was then placed back to its normal position and the peritoneum was sutured using an absorbable suture (Resorba). Isoflorane inhalation tube was removed and the mouse was monitored over the heated-pad plate to recover. After surgery analgesic used was paracetamol (Dafalgan- kinder syrup-7ml in 60ml drinking water). Stiches were removed after 10 days and the experiment was terminated after 4 weeks. Mice were euthanized and kidney was carefully dissected for analysis.

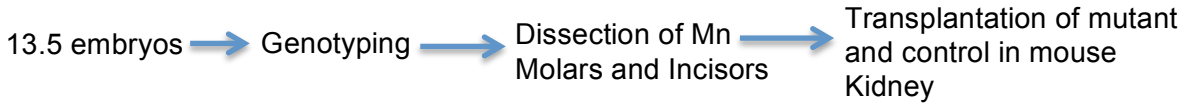


Figure 5.1 Schematic of KCT

5.5 Tissue preparation and Histology

Control and mutant embryonic heads; mandible; and KCT samples were fixed in 4% PFA (paraformaldehyde). Samples older than postnatal day 5 and KCT were transferred to 70% Ethanol for micro CT analysis, rehydrated and decalcified using 10% Ethylenediaminetetraacetic acid (EDTA) solution for at least a week at 4C. Adult mandibles were decalcified for up to 1 month before processing for embedding in paraffin wax. For embedding (embryonic heads) in OCT (Optimal Cutting Temperature), after fixation the samples were equilibrated with 30% sucrose in PBS (Phosphate Buffered Saline) at 4C. Tissue orientation used was frontal to examine molar and incisor tooth.

Paraffin blocks were cut on a microtome (model- `820`Spencer Microtome) and sections placed in an oven at 60˚C for 30 min. and stored at room temperature until use. For histological or immumnochistochemical analysis sections were de-paraffinized in xylol, and rehydrated in a series of decreasing gradient of ethanol solutions following staining with Haemotoxylin and Eosin or used for Immunohistochemistry or immunofluorescence.

5.6 Immunohistochemistry

Embryo heads and KCT (Kidney capsule transplantation) samples fixed in 4% PFA (paraformaldehyde), embedded in paraffin wax sections were deparafinized using series of gradient ethanol washes followed by boiling in 10mM sodium citrate buffer (pH 6) for antigen retrieval. To inactivate endogenous peroxidases sections were washed with 6% H_2O_2 in methanol for 20 minutes. After this the sections were incubated with primary antibody at 4°C overnight. The primary antibodies used were: anti-Dentinsialoprotein (DSP, rabbit, 1:200, Santa Cruz) anti-Amelogenin (rabbit, 1:1000, Abcam) Nestin (1:100 abcam), Phosphor-Smad1/5/8 (Cell signaling 1:200), Bonesialoprotein (1:100, abcam), Notch 1(1:100 Mitsiadis Lardelli et al 1995), anti-Pax9 (7C2) (1:400 abcam), non phosphorylated (active) β-catenin (1:1000 Cell Signalling), Bmp2/4 (1:400 Peprotech Inc). A secondary, anti-rabbit, and anti-rat biotin- conjugated antibody (1:2000, Southern Biotech) was used for the

immunohistochemistry.

5.7 RNA sequencing analysis

P0 lower molars were dissected from mutant and control *Bmp2* mice. RNA was extracted using Thermo Scientific extraction kit. 1µg of samples from both control and mutants were deposited to FGCZ Centre Zurich. Functional Genomics Center Zurich (FGCZ) performed Microarray and Bioinformatics analysis on the samples submitted.

5.8 In situ hybridization

RNA in situ hybridization (ISH) was performed on cryosections or paraffin sections (Mitsiadis et al., 1995). Standard procedure was used to prepare single stranded antisense digoxigenin-UTP labeled RNA probes. Plasmid vector was linearized using restriction enzymes. Labelled probe was purified using ethanol precipitation method, resuspended in 100mM DTT and diluted in hybridization solution (60% deionized formamide, 20 mM Tris-HCl, 5 mM EDTA, pH 8, 0.3 M NaCl , 0.5 mg/ml yeast RNA, 5% dextran sulfate). Sections were incubated with probes overnight in a humid chamber at 60C. The following day washing was performed and the sections were incubated in blocking solution (MABT+ 20% Normal Goat Serum) for 30min. After this the sections were incubated with Anti-digoxigenin (DIG)- AP (Alkaline Phosphatase conjugate) Fab fragment (Boehringer Mannheim, 1093 274) diluted 1:1000 in blocking solution. NBT (Nitro Blue Tetrezolium-Sigma 6876) and BCIP (5-Bromo-4-Chloro-3-Indolyl Phosphate- Sigma 8503) in staining solution (NaCl 2%, $MgCl_2$ 5%, Tris-HCl pH 9.5 10%, Tween-20 1% and Levamisole 0.5%.) were used to develop a color reaction. The sections were monitored every 2 hours for staining and were fixed in PFA 4% overnight to stop the reaction and mounted in Mowiol.

5.9 Skeletal preps

Embryos were skinned, neatly eviscerated and fixed in 95% ethanol for few days following incubation in acetone for 2 days to remove fat. Staining with Alizarin red and Alcian blue in glycerol and ethanol was performed for 3 days protected from light. After the staining,

sample was cleared in 1%KOH solution for at least 48 hours following clearing in sequential series of glycerol concentrations (20% glycerol in 1% KOH; 50% glycerol in 1% KOH; 80% glycerol in 1% KOH). Stored in 100% glycerol protected from light.

5.10 Scanning Electron Microscopy (SEM)

The KCT tooth were isolated and dissected from the kidneys after 4 weeks. This was processed for Backscatter imaging or SEM. Samples were prepared by fixing them in 4%PFA for 2 days. After this the samples were washed in buffer solution and dehydrated in series of ascending grades of alcohol and was fixed in Technovit 7200 VLC. Undecalcified sections were prepared using a cutting and grinding system (as described in Wolff et al., 2010). Processing of the sample and imaging was performed by electron microscopic facility staff at Oral Biology Institute in University of Zurich. SEM analysis was used for high resolution imaging of the KCT tooth.

5.11 RNA extraction and RT-PCR

Total RNA was extracted using Thermo Scientific RNA extraction kit and cDNA was transcribed from 0.5-1µg RNA using Thermo Scientific cDNA kit. Quantitative RT-PCR was performed using appropriate primer pairs and SYBR green was used for detection. The primers used are listed in Table 5.1. For each reaction 10ng/ul of cDNA was used. A housekeeping gene 36B4 was used as a control and relative expression of each of the gene was calculated by $Ct_{\text{candidate gene}}$-$Ct_{36B4}$ as described in Schmittgen et al., 2000). Analysis was performed on triplicate samples. For each reaction 10µl of 2X SYBR green master mix was mixed with 8µl of RNase free water, 1µl of primer and 1µL of cDNA. The plate was covered with adhesive cover (Thermo Scientific) and spun for 3 minutes 500 rpm. The plate was analyzed by RT-PCR machine (Bio-rad).

Bmp2 forward	GGAAGACGTCCTCAGCGAAT
Bmp2 reverse	ACGGCTTCTTCGTGATGGAA
Bmp3 forward	CAAAATGTTCTCCGACTTGGT
Bmp3 reverse	GTAACACGGTCCGCAGCTT
Bmp4 forward	GAGCCAACACTGTGAGGAGT
Bmp4 reverse	ATACGGTGGAAGCCCTGTTC
Bmp5 forward	AGATCTGGGATGGCAGGACT
Bmp5 reverse	GCTTGGGAACATGGTCTGGA
Bmp6 forward	CATGAGCTTTGTGAACCTGG
Bmp6 reverse	CCCTCAGGAATCTGGGATAG
Bmp7 forward	CGCCCATGTTCATGTTGGAC
Bmp7 reverse	ATGGTGGTATCGAGGGTGGA
Follistatin forward	TGCTGCTACTCTGCCAGTTC
Follistatin reverse	TGCTGCAACACTCTTCCTTG
Noggin forward	TGGTGTGTAAGCCATCCAAG
Noggin reverse	GGAAATGATGGGGTACTGGA
Twg forward	TGACGTTCCTGCTGTGTCTC
Twg reverse	AGCATGCACTCCTTACAGCA
Chordin forward	GCCATGACACTGGAGACCAA

Chordin reverse	ATCTGTCATAGCGGGCACTG
Grem1 forward	CAGCTGTTGGCAGTAGGGTC
Grem1 reverse	ACAGCGAAGAACCTGAGGAC
Grem2 forward	GCTACCATCCTTGTAAGGCG
Grem2 reverse	AGGCTTCCATCTCGTCATTG
Axin2 forward	GCAGAAGCCACACAGAGAGT
Axin2 reverse	CACCTCTGCTGCCACAAAAC
Lrp5 forward	TGCTGTACTGCAGCTTGGTC
Lrp5 reverse	CTACAGCAGCTGCATCCTCC
Wnt10a forward	CCACTCCGACCTGGTCTACTTTG
Wnt10a reverse	TGCTGCTCTTATTGCACAGGC
Lef1 forward	GTCCGAAATCATCCCAGCCA
Lef1 reverse	CTTGTTGTACAGGCCTCCGT
Wntless forward	TTTGGTGACATCCGACAGGG
Wntless reverse	CTTGCTTCCAGTACCCTGCA
Lgr5 forward	AGGCTGCCAAAAACTTCAGA
Lgr5 reverse	TAACCCAGTCACAGGGAAGG
Dkk1 forward	CCGGGAACTACTGCAAAAAT
Dkk1 reverse	GTCAGTGTGGTTCTTCTGGGA
Wif1 forward	CCATCAGGCTAGAGTGCTCA

Wif1 reverse	GCATTCTTTGTTGGGCTTTC
Hes1 forward	TGAAGGATTCCAAAAATAAAATTCTCTGGG
Hes1 reverse	CGCCTCTTCTCCATGATAGGCTTTGATGAC
Sostdc forward	AGCTCCTTCAGAGGGCTGAT
Sostdc reverse	GAGGCAGGCATTTCAGTAGC
Sost forward	TGTCAGGAAGCGGGTGTAGT
Sost reverse	CCTCCTCCTGAGAACAACCA
Mmp9 forward	AGACGACATAGACGGCATCC
Mmp9 reverse	CTGTCGGCTGTGGTTCAGT
Mmp12 forward	ATGAGGCAGAAACGTGGACT
Mmp12 reverse	TTTGGATTATTGGAATGCTGC
Mmp20 forward	TGTGGAGTTCCTGATGTGGC
Mmp20 reverse	GACAGCTGTACTCCACGCAT
Klk4 forward	GCCTCATCCTTGAGGTCACA
Klk4 reverse	CAAGACTCCCGAGCAGAAAA
K14 forward	CTGAAGAGCAAGATCCTGGCA
K14 reverse	TTCTTCAGGTAGGCCAGCTCC
Amlx forward	TTTGCTATGCCCCTACCACCT
Amlx reverse	GTGATGAGGCTGAAGGGTGTG
Ambl forward	CAAAGCATCCGCTTTTTACC

Ambl reverse	GCCTCCAAATCTTGGGAACAG
Dcpp1 forward	CCCCACCGTTTTGACACAAG
Dcpp1 reverse	AGCCATACACTTGACCGTCC
Dcpp2 forward	CCCCACGGTTTTGACACAAG
Dcpp2 reverse	CTTGACCGTCCTCGTTGTCA
Dcpp3 forward	CCCCACCGTTTTGACACAAG
Dcpp3 reverse	GCCATACACTTGACCGTCCT
Aqp5 forward	TGAACCCAGCCCGATCTTTC
Aqp5 reverse	CTCAGCGAGGAGGGGAAAAG
Muc19 forward	AGAACCACCAGTATGCCAGC
Muc19 reverse	GACCATGACACACTGGGGTT
Dspp forward	AGCAGTGAGGAAAACGGTGT
Dspp reverse	TGTTGCCTTTGTTGGGACCT
Nestin forward	AGCAGGAGAAGCAGGGTCTA
Nestin reverse	AGGTGCTGGTCCTCTGGTAT
Dmp1 forward	CCCAGTTGCCAGATACCACA
Dmp1 reverse	TCTGTACTGGCCTCTGTCGT
Sp7 forward	GATGGCGTCCTCTCTGCTT
Sp7 reverse	CGTATGGCTTCTTTGTGCCT
Alpl forward	ACAGACCCTCCCCACGAGT

Alpl reverse	TGTACCCTGAGATTCGTCCC
OC/Bglap1 forward	TGACAAAGCCTTCATGTCCA
OC/Bglap1 reverse	ATAGCTCGTCACAAGCAGGG
OPG forward	CCACAATGAACAAGTGGCTG
OPG reverse	TCACACAGGAGCTGATGACC
Runx2 forward	ACACCGTGTCAGCAAAGC
Runx2 reverse	GCTCACGTCGCTCATCTTG
Col1a1 forward	TGTCCCAACCCCCAAAGAC
Col1a1 reverse	CCCTCGACTCCTACATCTTCTGA
Bsp forward	GACGGCGATAGTTCCGAAGA
Bsp reverse	TGCATCTCCAGCCTTCTTGG
Bsp I forward	AATCTCCTTGCGCCACAGAA
Bsp I reverse	GCAGTGACGGTCTCATCAGA
Pitx2 forward	CCTCCTCACCCTTCTGTCACC
Pitx2 reverse	GCCCACATCCTCATTCTTTCC
Pax9 forward	GTCACGGACATTCTGGGCAT
Pax9 reverse	CATGCTGGATGCTGAGACGA
Barx1 forward	CGAAAGCCAAGAAAGGACGC
Barx1 reverse	GCCACCTTGCAGCACTATTT
Tbx1 forward	TTGTGTTTGAGGAGACACGC

Tbx1 reverse	AGCGTCTTTGTCTGAGCCAT
Msx1 forward	TCTCGGCCATTTCTCAGTCG
Msx1 reverse	AGCTGAGCTGTGGTGAAAGG
Sox2 forward	CGTAAGATGGCCCAGGAGAAC
Sox2 reverse	CCGGGAAGCGTGTACTTATCC
Sox9 forward	CTCAGCAAGACTCTGGGCAA
Sox9 reverse	TCGGGGTGGTCTTTCTTGTG
Mmp13 forward	CTTCTGGCACACGCTTTTCC
Mmp13 reverse	TCCTGGGTCCTTGGAGTGAT
Nestin forward	AGCAGGAGAAGCAGGGTCTA
Nestin reverse	AGGTGCTGGTCCTCTGGTAT

Table 5.1- List of qPCR primers

5.12 Statistical Analysis

RT-PCR and quantitative data are presented as mean ± SD. Errors bars show Standard deviation. Analysis between groups was performed using unpaired t-test using Microsoft excel. *P* value <0.01 was considered to be statistically significant. For RT-PCR analysis at least two independent groups were performed in triplicates. Number of repeats of each independent experiment is denoted as n.

6. Results

6.1 *Bmp2* Expression in adult and developing tooth

To analyze the expression of *Bmp2* in postnatal tooth we used a *Bmp2 lacZ* reporter mouse (Chandler et al., 2007). For embryonic stages *Bmp2* expression was detected using in situ hybridisation at transcriptional level and immunohistochemistry at translational level. In an adult one-month-old mandible *Bmp2* was expressed in and around the roots (Figure 6.1C), gingiva and periodontal ligaments (Figure 6.1D) and in the pulp (Figure 6.1F). Also *Bmp2* was expressed inside the incisors (Figure 6.1E). This shows that *Bmp2* is expressed in most of the major structures of the adult tooth indicating that *Bmp2* might be important in tooth homeostasis life long.

Expression analysis of Bmps (Aberg et al 1997) has shown an interesting *Bmp2* expression pattern, where *Bmp2* is expressed in the epithelium at early stages of tooth development up to the early bell stage and at E18.5 the expression shifts from epithelium to mesenchyme where it is also detected at PN1. We confirmed some of the previous expression data published using in situ hybridisation. During early stages *Bmp2* expression was indeed confined to the epithelium (Figure 6.2D) and later at early mineralization stages around E18.5 *Bmp2* expression was clearly expressed in the mesenchyme (Figure 6.2C). Since focus of our study were the early mineralization stages we focused our expression data at P0 of mouse tooth development where epithelium derived enamel-forming ameloblasts and mesenchyme derived dentin-forming odontoblasts cells are differentiating and some limited mineralization has occurred in first molars. In situ hybridization for *Bmp2* at P0 (mineralization stage) revealed *Bmp2* expression mainly in odontoblasts (Figure 6.2A) with a small fraction of cells expressing *Bmp2* in ameloblasts (Figure 6.2B).

Bmp2/4 localization at protein level was done by immunohistochemistry. As mature forms of Bmp2 and Bmp4 in mouse are identical, human Bmp2 and Bmp4 are 98% identical. We considered using the anti-Bmp2 antibody and not Bmp2-specific as it likely detects both Bmp2 and Bmp4. Expression was seen both in ameloblasts and odontoblasts (Figure 6.3).

At P0 stage protein expression appeared localized mainly between the two cell layers suggestive of directed secretion and signaling (Figure 6.3).

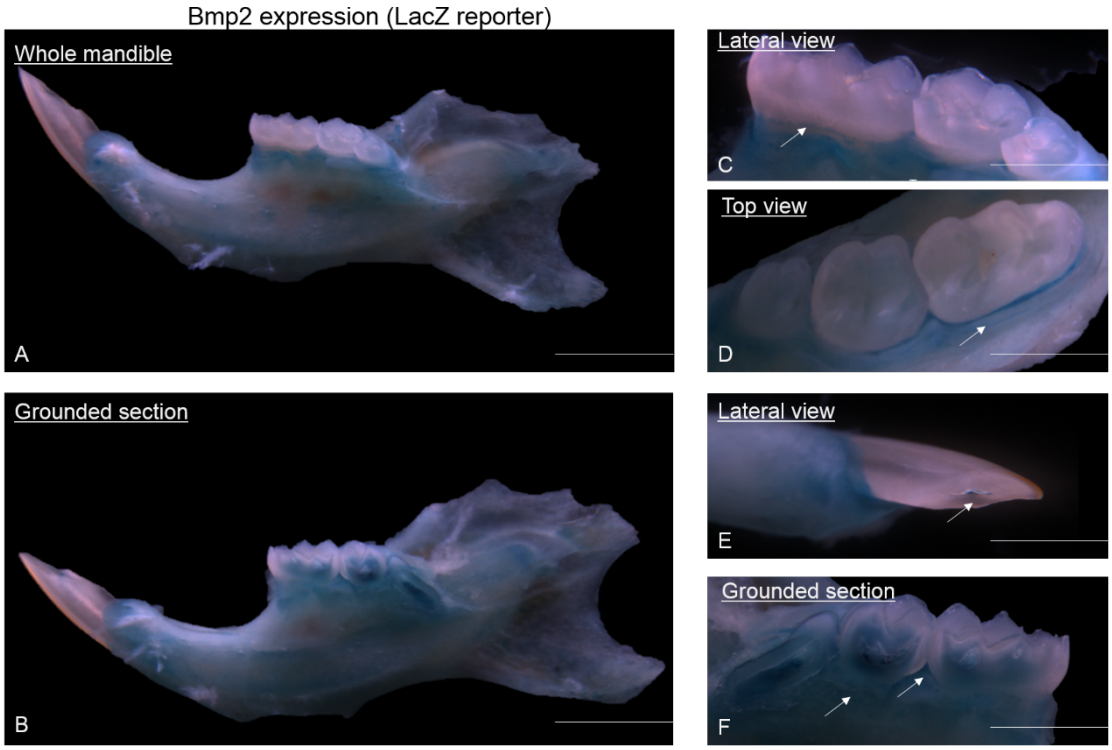


Figure 6.1: Bmp2 expression in adult 1-month-old lacZ stained mandible. (A) Whole mount x-gal staining of Bmp2-lacZ stained mandible. (B) Grounded section exposing the lacZ stain in the pulp. (C) Lateral view of the molars showing expression in and around roots (arrow). (D) Top view of the molars showing expression in the gingiva and periodontal tissue (arrow). (E) Lateral view showing expression of Bmp2 inside the incisors (arrow). (F) Grounded section of the molars showing prominent Bmp2 stain in the pulp and gingiva (arrows). Scale bar A-B: 2mm, C-F: 1mm.

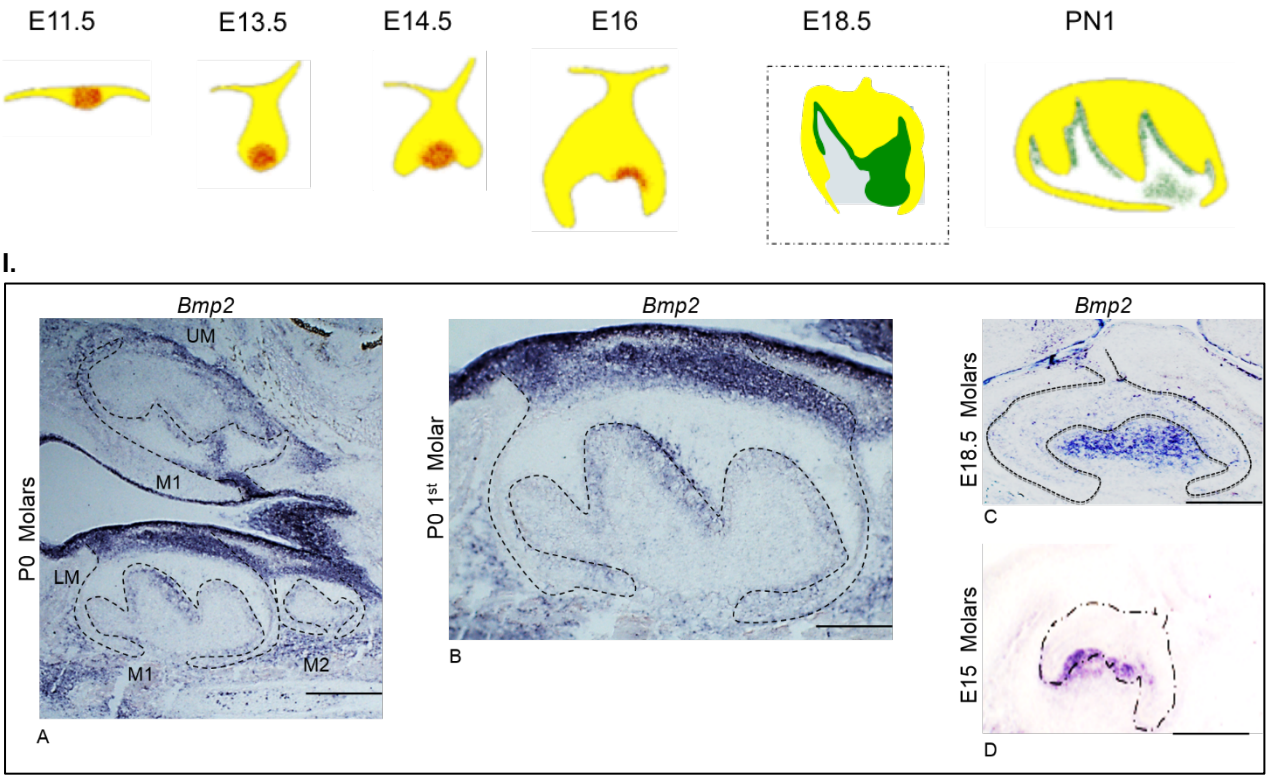


Figure 6.2: In situ hybridization of mouse molars during different stages of development showing *Bmp2* expression. **(I.)** *Bmp2* expression data depicting shift from epithelium to mesenchyme (Modified from Aberg et al 1997) **(II.)** (A) Sagittal section of postnatal day 0 (P0) mouse molars. (B) Sagittal section of P0 mandibular 1st molar showing asymmetric *Bmp2* expression. (C) Frontal section of E18.5 molar showing expression mainly in the mesenchymal derivative of tooth. (D) Frontal section of E15 molar showing expression mainly in the epithelial derivative of the tooth. Note the shift from epithelium to mesenchyme from E15 to E18.5. Scale bar A: 500µm, B: 200µm, C: 100µm, D: 50µm.

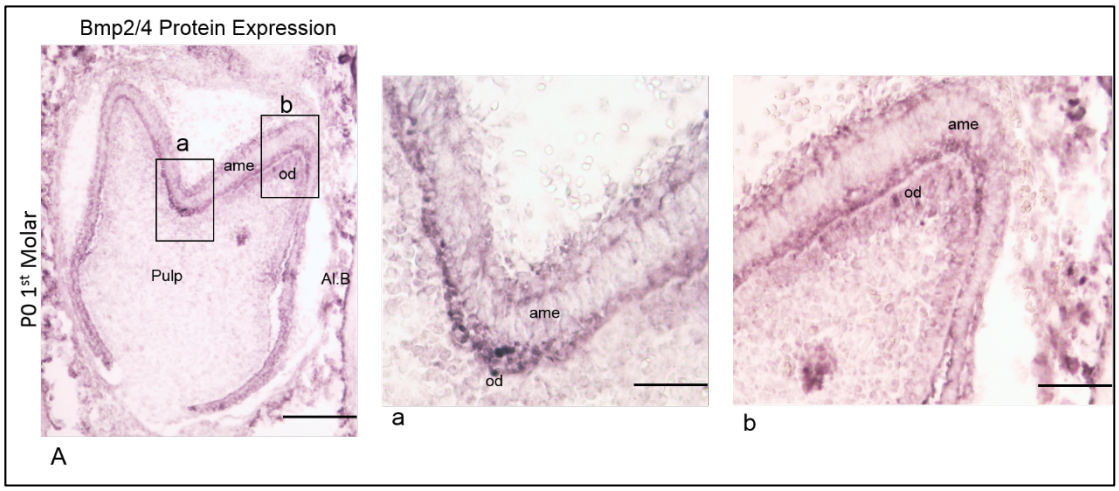


Figure 6.3: Bmp2/4 protein expression at P0. (A) Frontal sections of P0 molar showing Bmp2/4 expression. (a,b) Bmp2 expression in ameloblasts (ame) and odontoblasts (od). Scale bar A: 100µm, a,b: 50µm

6.2 Part I: *Bmp2* deletion in tooth epithelium

6.2.1 K14-Cre driver to study *Bmp2* deletion in epithelium

In order to study the phenotypic effects of *Bmp2* deletion in epithelium it is important to select a Cre driver with efficient deletion in oral epithelium already at early embryonic stages (E12.5, E13.5). For this, we crossed a K14-Cre allele to the Rosa26/lacZ reporter allele and performed lineage tracing at early stages of tooth development (Figure 6.4 A, B, C, D, E) as well as later stages (Figure 6.4F) to assess recombination. We found that K14-Cre targets both upper and lower dental placodes as early as E12.5, which was reflected by predominant marking of dental epithelial cell layers in P0 incisors. This establishes that our K-14 Cre allele induces recombination in oral epithelium from at least E12.5 onwards.

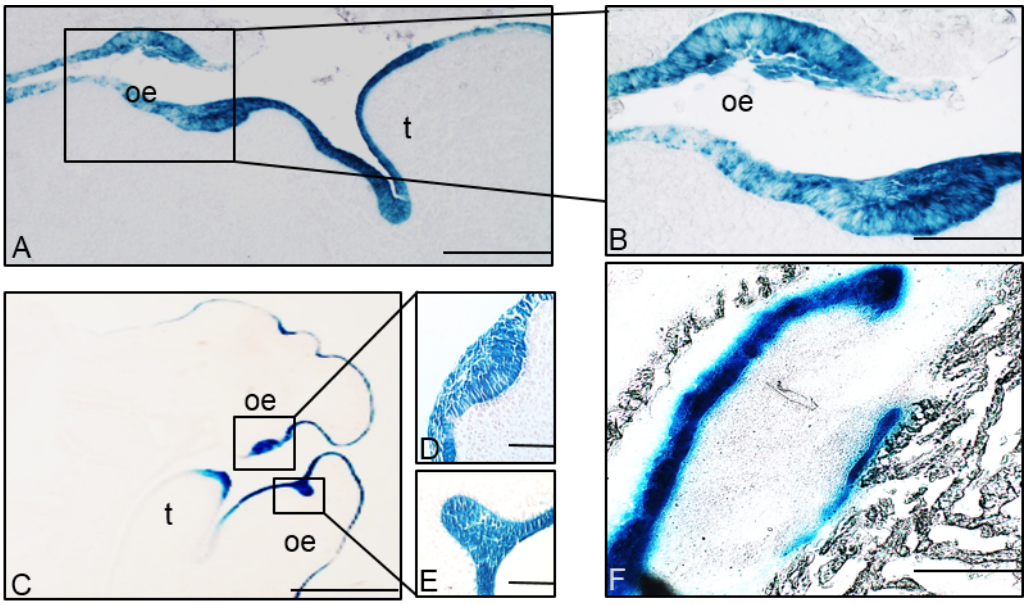


Figure 6.4: K14-Cre targets oral epithelium from early stages of tooth development: To study the effects of loss of *Bmp2* in oral epithelium we used a K14-Cre driver to ablate *Bmp2* specifically in the tooth epithelium. Lineage tracing shows that K14-Cre targets oral epithelium at early stages of tooth development (A and B: E12; C: E13; D, E magnification of C), which is reflected at P0 in a longitudinal section through a lower incisor (F). Note the both upper and lower dental placodes are targeted by K14-Cre. Abbreviation: oe- oral epithelium; T- tongue. Scale Bar (C,F): 200µm; (B,D,E): 50µm; (A):100µm

6.2.2 Phenotypic Characterization of *Bmp2* deletion in epithelium

Deletion of *Bmp2* in epithelium using K14-Cre results in viable mice appropriate for studying adult teeth. *Bmp2*-deficient mice were identified by genotyping. The conditional mutants (cKO) (*Bmp2* deficiency in epithelium) were slightly smaller at 3 months of age (Figure 6.5A,B) with a receding hairline phenotype. The slight difference in weight could be due to improper feeding and not efficient enamel in cKO mice. Due to limitations, only teeth were analysed and other phenotypes such as the hair phenotype were not studied further.

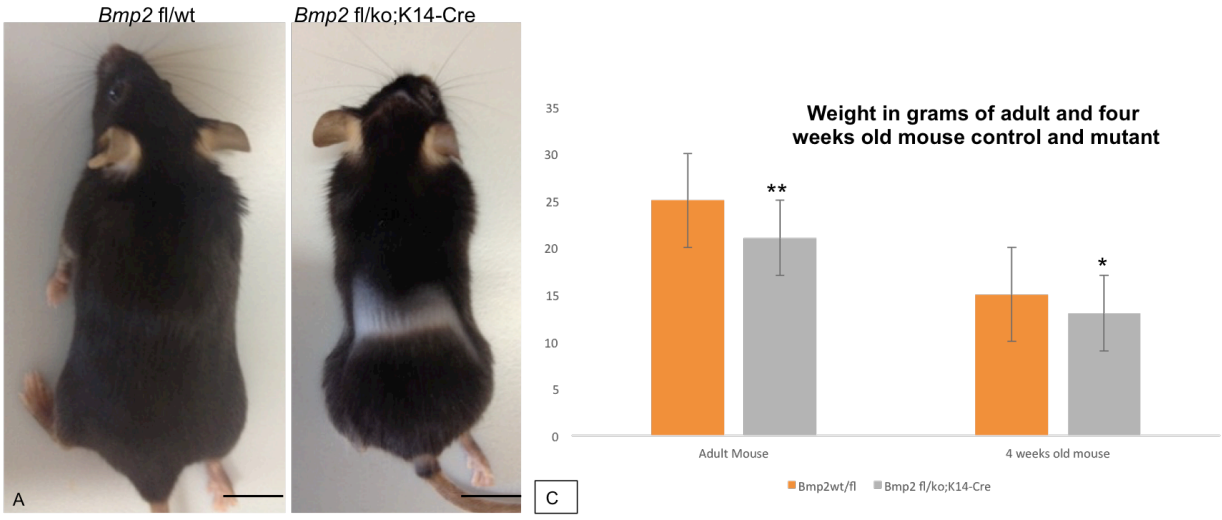


Figure 6.5: *Bmp2* deletion in epithelial cells using K14-Cre. (A, B) Adult 3 months old mice showing differences in mutant ((B) *Bmp2* fl/ko;K14-Cre) and control ((A) *Bmp2* fl/fl). Note the receding hairline and a smaller size of the mutant (B). (C) Comparison of weight of the mutant and control mice between 4 weeks and adult (3 months old mice) (** *P*<0.001) (**P*<0.01) show significant differences (n=5). Note reduction in weight of the mutant mice.

6.2.3 Gross morphology of teeth from *Bmp2* deficient epithelium

Bmp2 deletion in oral epithelium did not affect tooth number with one incisor and three molars in each quadrant of the mouse jaw in 2-month-old mice (Figure 6.6 A, A`) or gross morphology. Size and structure of the mandible of cKO looked fairly normal (Figure 6.6 A, A`) without any noticeable differences. Close inspection of the incisors did reveal some differences in thickness of the incisors (Figure 6.6 B, B`). Molars showed no noticeable difference except for a slight change in cusp pattern. Cusp pattern of the cKO molars were

more flat when compared to wild type (Figure 6.6 C, C`), but differences in cusp patterns were not analyzed in detail.

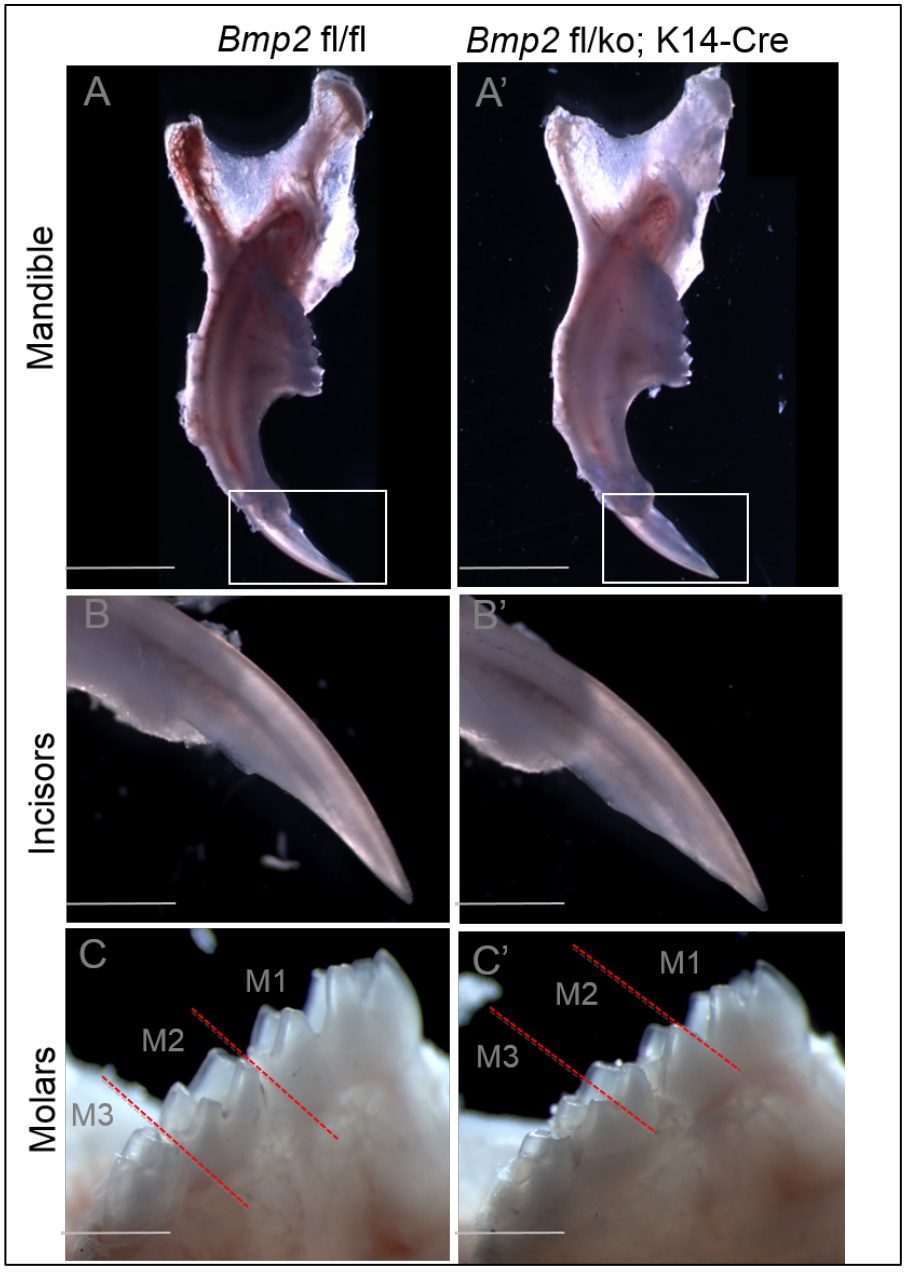


Figure 6.6: Phenotypic analysis of 2 month old mice with K14-Cre mediated deletion of *Bmp2* (A, A') Mandible of the mutant (A') and control (A) with formation of both incisors and molars in quadrant of each jaw. Shape of the mutant incisor (B') is different as compared to the wild type incisor (B) Cusps of control molars (C) appear more prominent when compared to the mutant (C'). Scale bar (A, A'): 2mm; (B,B' and C,C'): 1mm

6.2.4 μ CT analysis revealed delayed mineralization in *Bmp2*-deficient teeth

μCT analysis was performed in order to address mineralization changes in molars and incisors of cKO mice. Analysis of serial frontal sections through the incisor revealed a delay in onset of mineralization of cKO incisors when compared to control mice (Figure 6.7 e, e`). Changes in mineralization were obvious at the level of third molars (Figure 6.7 a, a`,b,b`) whereas mineralization appeared unaffected at the level of 1st and 2nd molars (Figure 6.7 c,c`d,d`). This indicated that loss of *Bmp2* affects early stages of enamel formation, which does however not appear to affect the final enamel product. No defect was observed in the fully matured enamel of molars except for more flat cusp shapes (as described above).

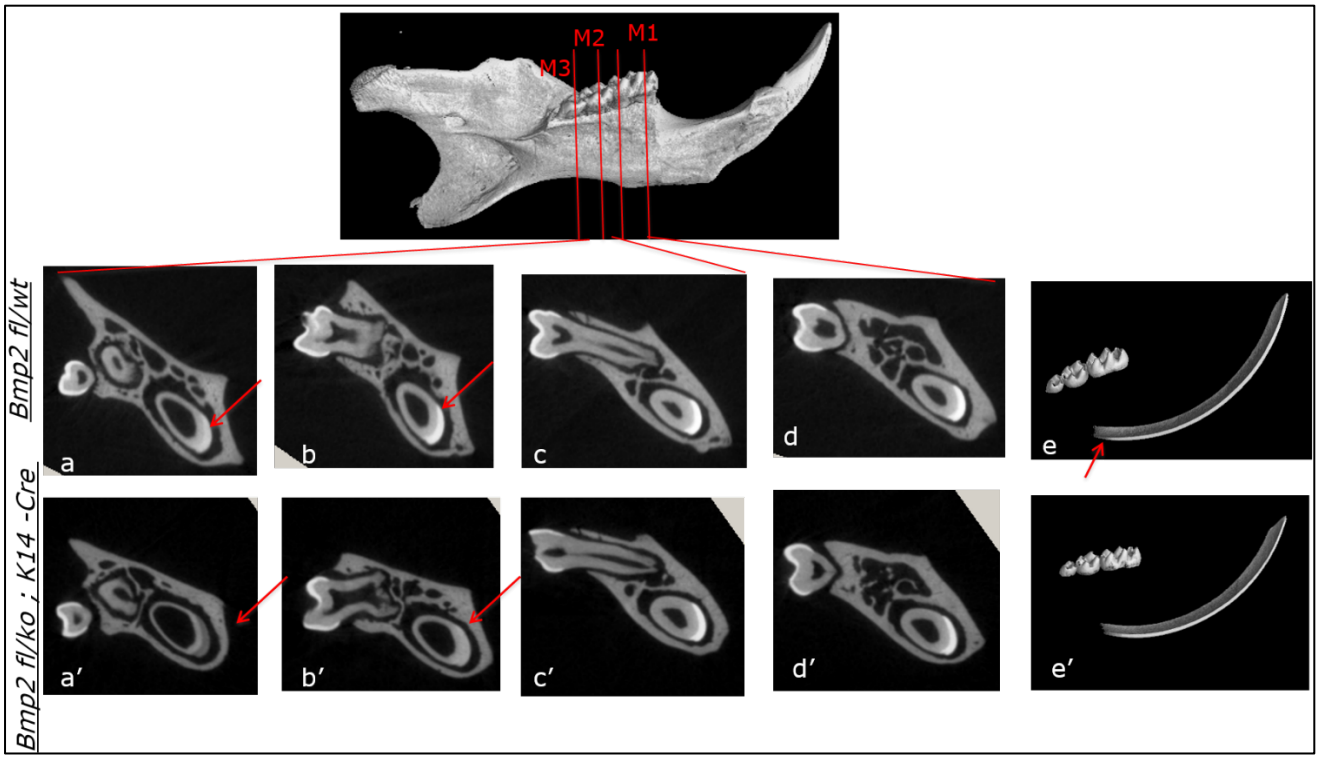


Figure 6.7: Delayed mineralization phenotype in the enamel of the mutant *Bmp2* incisor as revealed by μCT analysis.
Serial frontal sections through the mouse incisor from 6 week old control (a-e) and mutant (a'-e') mice shows a delay in mineralization in the mutant when compared to the control. Note- No change in molar mineralization was observed. Abbreviations: M1, M2, M3- Molar 1,2,3

6.2.4 Altered epithelial cell layer in *Bmp2* deficient mice

In order to investigate if the delayed mineralization phenotype of incisors was related to disruption of any of the epithelial layers we performed histological staining. Haemotoxylin and Eosin (H&E) stain revealed that the epithelial derived Stratum intermedium (SI) layer of the incisors was somewhat disorganized (Figure 6.8 B, B`). The Stratum intermedium layer is present during tooth development in both molars and incisors. Longitudinal sections of continuously erupting adult incisors allow observation of the different developmental stages of ameloblast differentiation, whereby the more immature cells are located posteriorly and more mature cells anteriorly (Wang et al 2004). Immunostaining of the epithelial layers showed altered expression of Amlx, which marks enamel matrix and Notch1, which marks SI (Figure 6.8 C,C`,D,D`). This suggests *Bmp2* is an important signal in organizing SI cells for proper enamel formation in incisors.

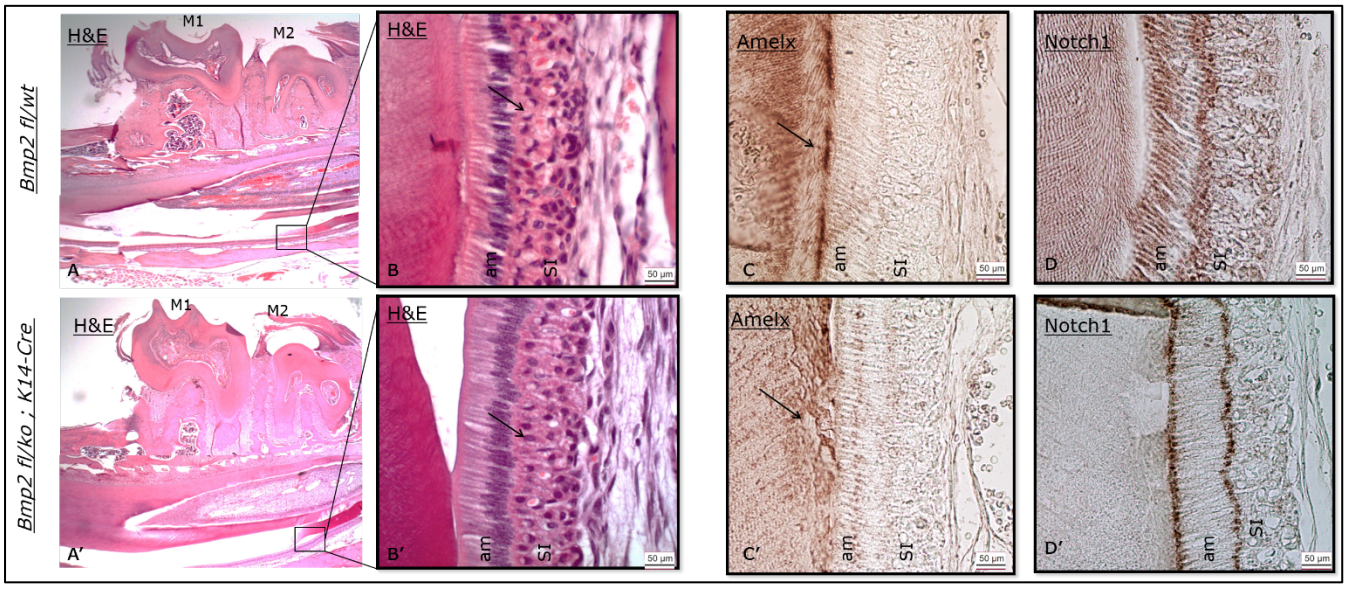


Figure 6.8: Altered cell morphology in the epithelial derived Stratum Intermedium in *Bmp2* mutants Histological analysis of control (A-D) and mutant (A'-D') decalcified incisor from 6 weeks old mice (A and A') shows different morphology of cells of the Stratum Intermedium (marked by arrows B and B') at the region where mineralization starts. Also, note the differences in the expression of Amlx in the enamel matrix in the mutant (C') when compared to the control (C'). Similarly, Notch1 expression in the stratum intermedium was altered (D and D'). Abbreviation: am-ameloblasts; SI-Stratum Intermedium. Scale Bar (A, A') 1mm; (B, C,D) and (B',C',D') 50µm
This may suggest that *Bmp2* helps to organize the underlying cells of the stratum intermedium, which is important for enamel mineralization.

6.3 Part II: *Bmp2* deletion in tooth mesenchyme

6.3.1 Phenotypic analysis of *Bmp2* deletion in Neural crest cells (NCC) using Wnt-1 Cre

Since to our knowledge no tooth- mesenchyme-specific Cre driver has been made, we used the well-established Wnt1-Cre driver-mouse to ablate *Bmp2* in all neural crest cells, from which dental mesenchyme cells are derived from (Ruch, 1990). Wnt1-Cre transgenic mice express Cre at E8.5 in premigratory neural crest cells (Chai et al., 2000) and lineage tracing studies using a gene-reporter mouse have shown that Wnt1-Cre activity marks oral mesenchyme at early stages as well as late stages of tooth development (Chai et al., 2000). *Bmp2* mutants were identified by genotyping. At all embryonic stages investigated, *Bmp2* fl/fl; Wnt1-Cre were found at the expected mendelian ratio. No *Bmp2* fl/fl; Wnt1-Cre mice survived to 8-10 days, the age when biopsies for genotyping were taken. Mutant *Bmp2* (*Bmp2* fl/fl; Wnt1-Cre) were found to be peri-natal lethal, which limited our investigation up to the P0 postnatal stage. We first investigated gross morphological differences in craniofacial head shape formation at P0. All craniofacial structures were formed in mutant mice, however the mutants had a slightly smaller head than their control littermates (Figure 6.9 A, A`). Both top view (Figure 6.9A, A`) and front view (Figure 6.9B, B`) revealed smaller head shape. This was confirmed by analysis of skeletal preparations, which additionally revealed a shorter mandible and apparent differences in bone density evident as different staining intensity by alizarin red (Figure 6.9 C, C`). Further µCT analysis revealed differences in the skull bones and sutural spaces between mutants and controls. (Figure 6.9 D, D`). A wider sutural space and shorter skull bones were found in mutant skulls compared to controls. These observations showed that neural crest-derived *Bmp2* is dispensable for neural crest formation and migration, as all major craniofacial structures are formed. These phenotypic alterations were not further pursued within the context of this thesis, but may be carried forward in a future craniofacial-specific project.

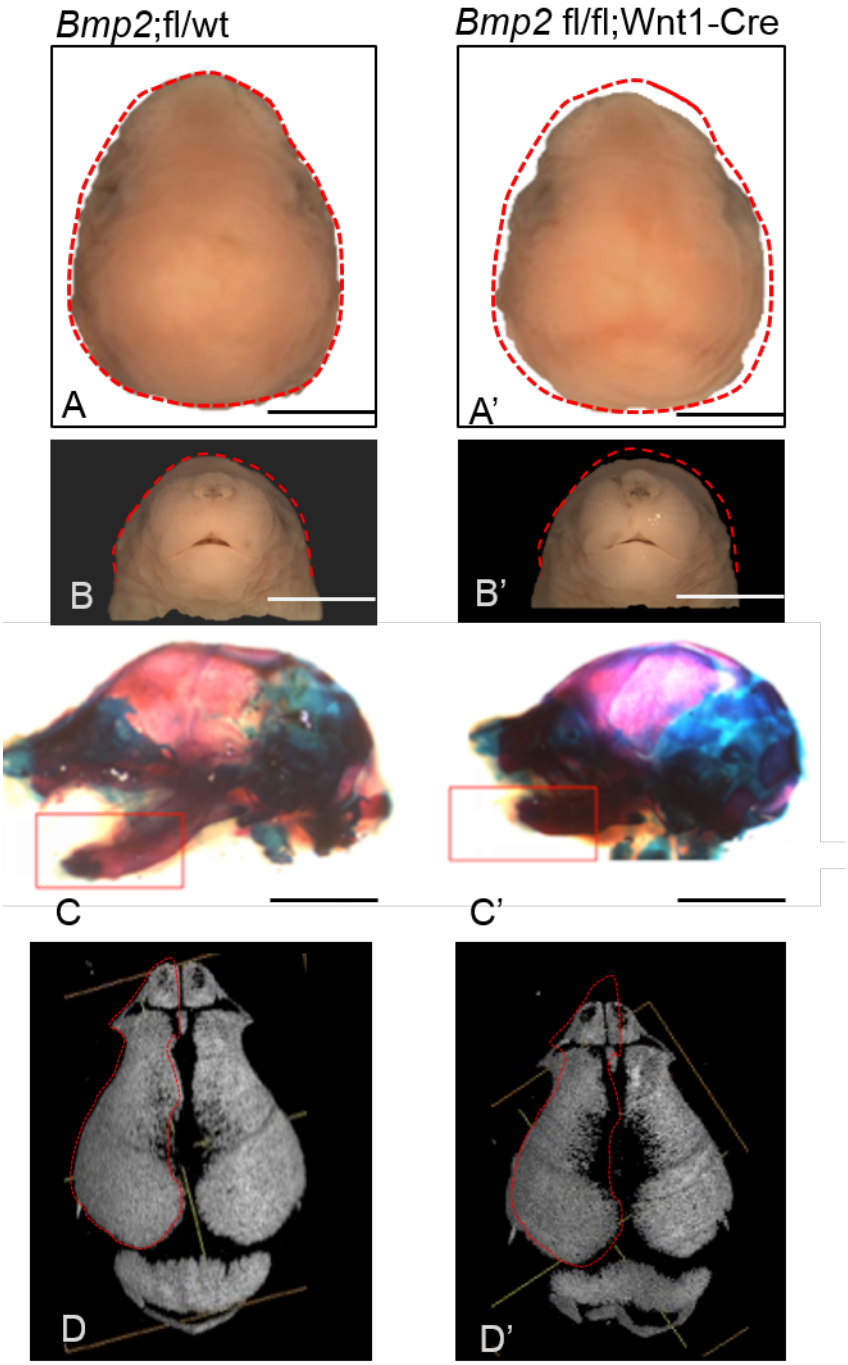


Figure 6.9: Comparison of gross morphology of heads of *Bmp2* fl/fl (control A-D) and *Bmp2* fl/fl;Wnt1- Cre (mutant A'-D') littermates at P0. (A, A') Top view showing smaller head of the mutant (A') as compared to the wild type (A). (B, B') Front view showing the mouth opening and nose structure. Note the differences in head arch (dotted line) between the mutant and control (C, C') Side view of the skeletal prep images reveals overall differences in mandibular size and shape (boxed), head shape and skull bones. (D, D'). Anatomical differences in skull bones and sutural space (dotted line) as shown by micro-CT imaging. Scale bar A-C, A'-C': 2mm.

6.3.2 μ CT analysis of *Bmp2* mutant

μCT analysis was performed on P0 heads (Figure 6.10 A, A`). This confirmed the overall head shape to be smaller in mutants as shown before (Figure 6.9 C). To further investigate the mandible phenotype, mandibles were digitally isolated using the cropping tool. 3D μCT isosurface images revealed differences in shape of the mandible (Fig. 6.10-11). Both bottom view (Figure 6.10 B, B`) and lateral view (Figure 6.10 C, C`) showed shorter mandible between the mutant and littermate control.

2D lateral cross-section μCT images revealed smaller incisor in *Bmp2* mutants (Figure 6.11 A, A`, B, B`). To assess whether a potential phenotype in the incisor volumetric analysis of both molar and incisors was performed. It revealed an overall decrease of length in mutant when compared to the wild type (Figure 6.11 C, C`). This data indicates that loss of *Bmp2* affects growth of mandible and possibly causes an incisor phenotype. Molars at this stage are not sufficiently mineralized to allow a similar volumetric analysis

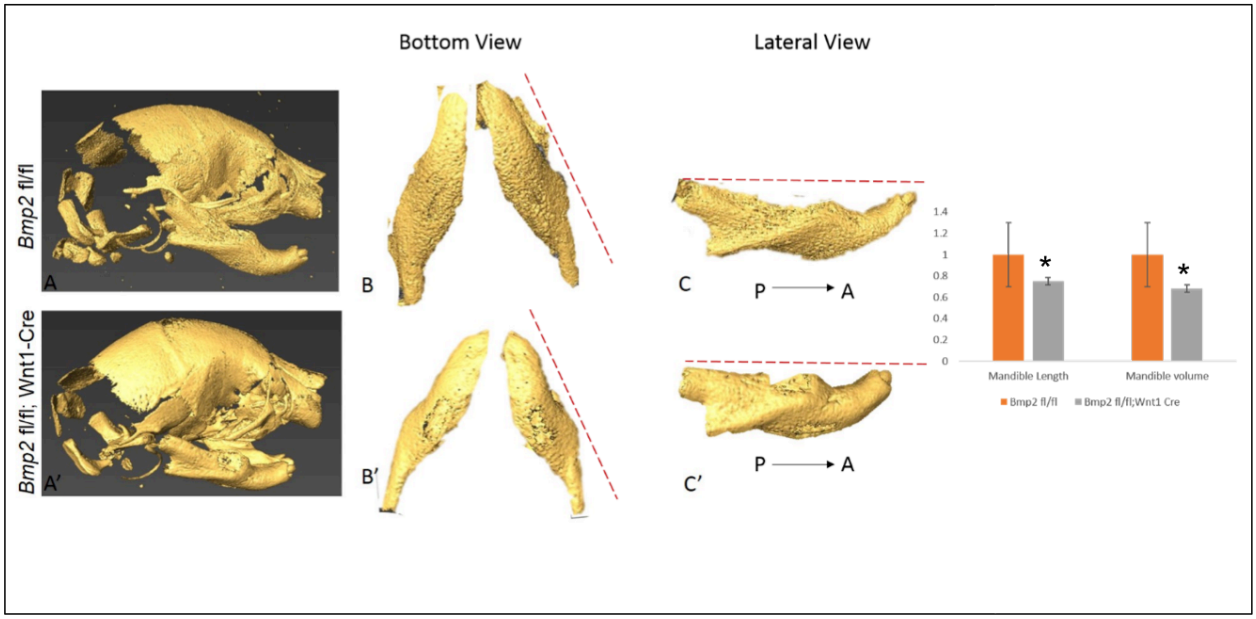


Figure 6.10: Comparison of the head shape and mandible shape using micro-computed tomography (μCT) analysis of *Bmp2* fl/fl (control A-C) and *Bmp2* fl/fl;Wnt1-Cre (mutant A'-C') littermates at P0. (A, A') Side view of the 2D μCT image of the head showing differences in the size of the head shape and mandible. (B,B' and C,C') Bottom and lateral view of the mandible showing difference in lengths of the mandible respectively (P→A –Posterior to Anterior axis). Histogram on right shows significant differences in relative volume and length of the control and mutant mandible (**P*<0.01) (n=2).

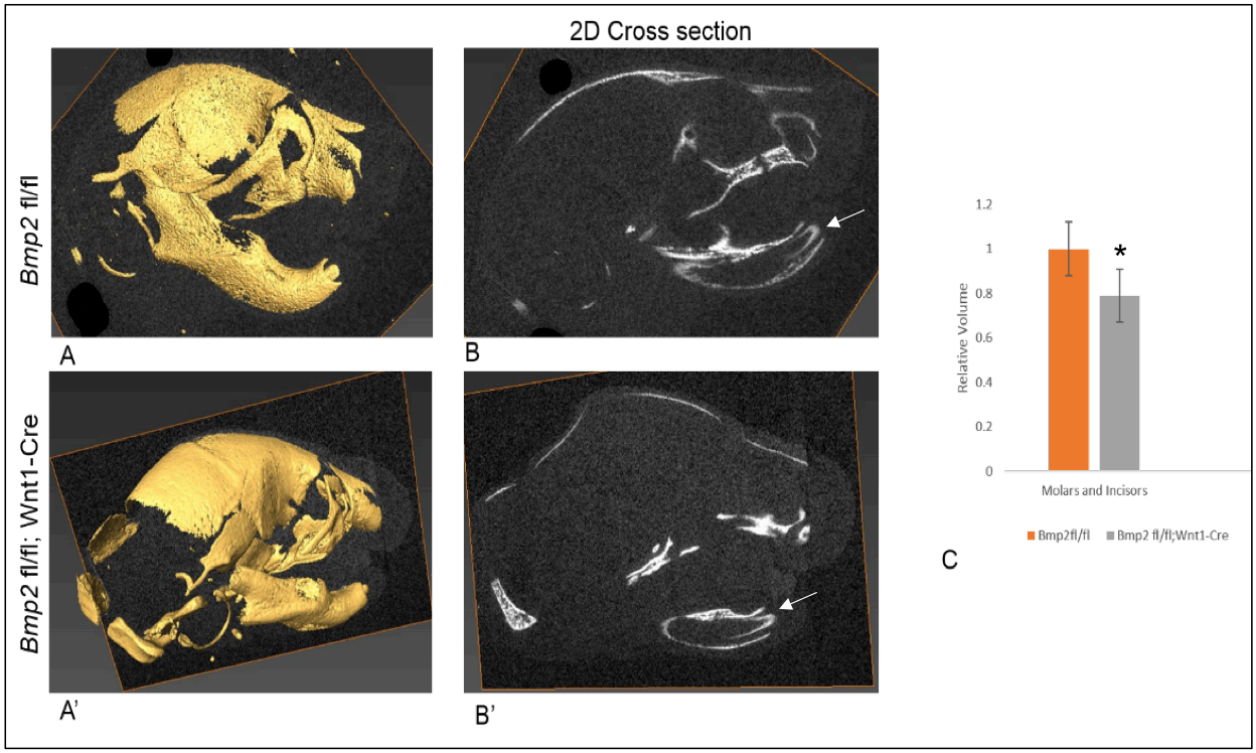
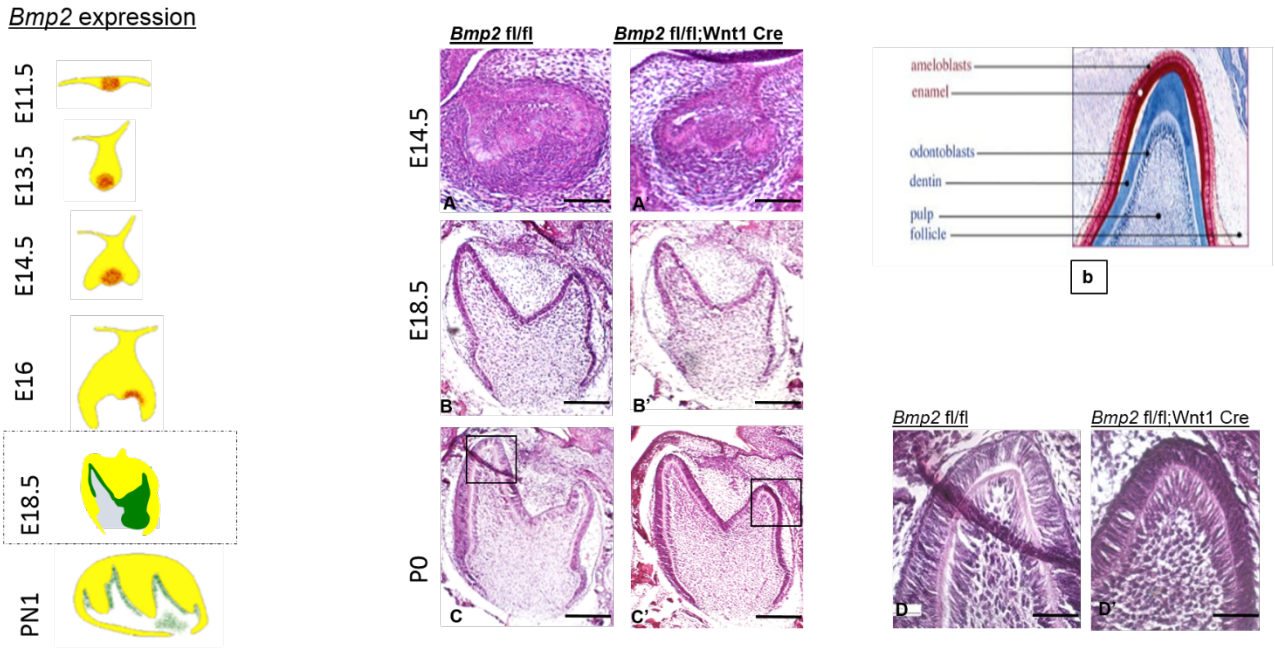


Figure 6.11: **Volumetric analysis of mandibular incisors and molars** of *Bmp2* fl/fl (control A-C) and *Bmp2* fl/fl;Wnt1-Cre (mutant A'-C') littermates at P0. (A, A' and B, B') 2D- µCT image and cross section plane showing the mineralized tooth structures (arrows) respectively. (C) Histogram showing relative volume of the control and mutant. Note significant reduction in the volume (*P<0.01) (n=2).

6.3.3 Deletion of *Bmp2* in tooth mesenchyme causes defects in odontoblasts and dentin matrix

To gauge whether loss of Bmp2 in tooth mesenchyme also affects development of molars, a histological analysis was performed. Frontal sections of P0 molars show an apparent delay in molar development evident as defects in odontoblast polarization and dentin matrix formation (Figure 6.12 D, D`, C, C). Tooth formation at earlier stages appeared unaffected; in line with our finding that mesenchymal expression of *Bmp2* is not observed prior to E18.5 (Figure 6.12 A, A`, B, B` and not shown). The shift of *Bmp2* expression from epithelium to mesenchyme (Figure 6.12 a) coincides with the onset of odontoblast and ameloblast differentiation, which starts at E18.5.



a.

Figure 6.12: Bmp2 during tooth development. (a) Illustration of Bmp2 expression during tooth development (Modified from (Aberg et al., 1997) Red: expression in oral epithelial derived cells, green: expression in dental mesenchyme derived cells. (b) Schematic of the different layers of tooth at P0. (A-B and A'-B') Deletion of *Bmp2* from neural crest-derived dental mesenchyme (Wnt1-Cre) did not affect tooth development before P0, as evident from H/E stained sections from *Bmp2* fl/fl control (A-B) and *Bmp2*fl/fl: Wnt1-Cre mutant mice (A'-B'). At P0, slightly disrupted development is evident in the mutant tooth (C',D') compared to the control (C,D). Analysis was performed on mandibular first molar frontal sections. Scale Bar A-C and A'-C': 100µm, D, D': 50µm

6.3.4 Molecular Analysis revealed no developmental delay until E18.5

To assess whether tooth development proceeds normal up to E18.5, the stage when *Bmp2* is first expressed in dental mesenchyme, we performed in situ hybridization at E18.5 for key epithelial and mesenchymal genes. None of the genes showed any obvious change in expression pattern (Figure 6.13 A, A`- F, F`) between mutant (Figure 6.13 A`-F`) and control (Figure 6.13 A-F). This indicates that molar tooth development proceeds normal up to E18.5 in mice with a neural crest-specific deletion of *Bmp2*.

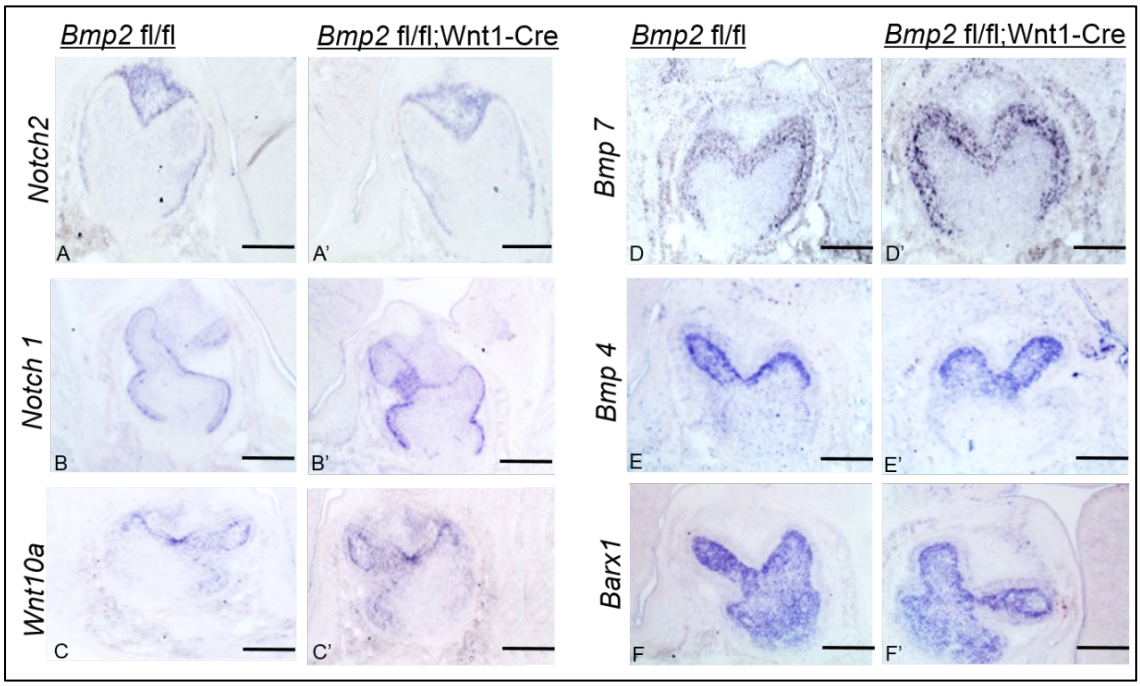


Figure 6.13: Gene expression at E18.5 (Bell stage). In situ hybridization for several genes involved in tooth development at E18.5 (frontal sections) revealed no discernible differences in their expression between teeth in which *Bmp2* was deleted (*Bmp2* fl/fl Wnt1-Cre, A'-F') in the mesenchyme and control teeth (*Bmp2* fl/fl, A-F), indicating that Bmp2 is dispensable up to this developmental stage. Scale bar (A,A')- (F,F'): 100µm

6.3.5 Proliferation and Apoptosis at P0

To test whether dentin matrix defects observed at P0 were the result of changes in proliferation or apoptosis we performed immunohistochemistry, focusing on the affected structures (areas where ameloblasts and odontoblasts form). Staining for Ki67 revealed that there was little or no proliferation in ameloblasts or odontoblasts, in contrast some proliferation in the dental pulp and the alveolar bone surrounding the tooth was observed (Figure 6.14 A, A`). Apoptosis was assessed staining for cleaved caspase-3, which revealed a few apoptotic cells in the ameloblasts layer of mutant and control teeth (Figure 6.14 B, B`). This indicates that the apparent delay in tooth mineralization is not due to proliferation or apoptotic defects.

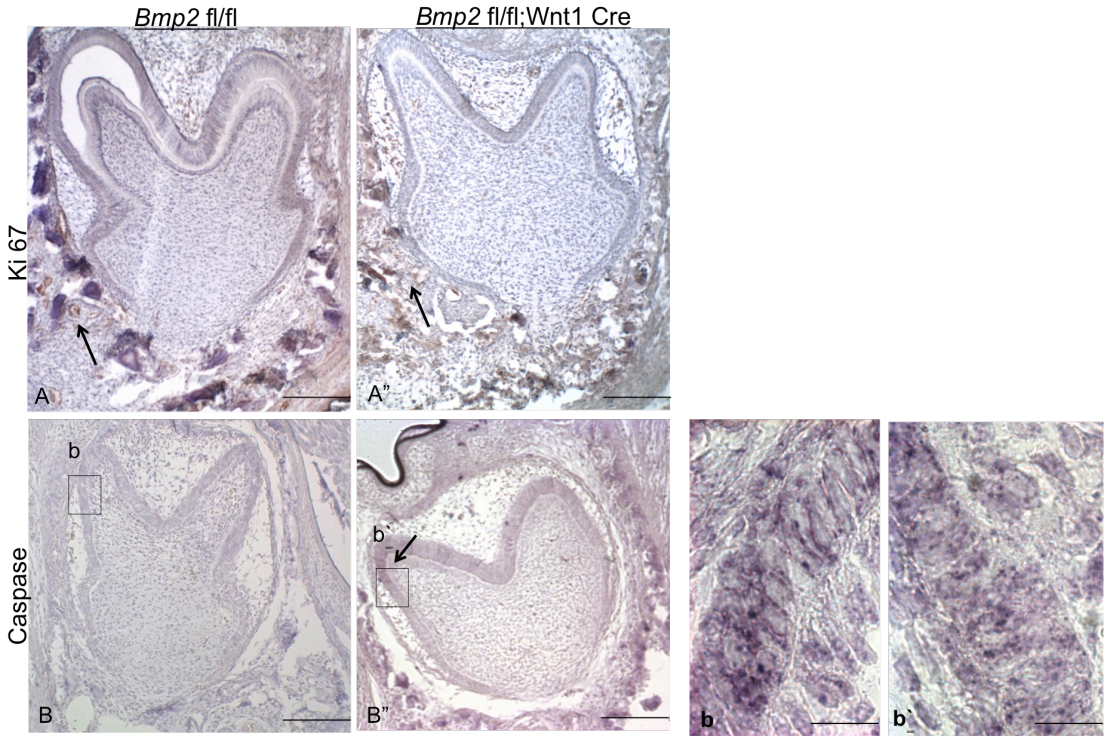
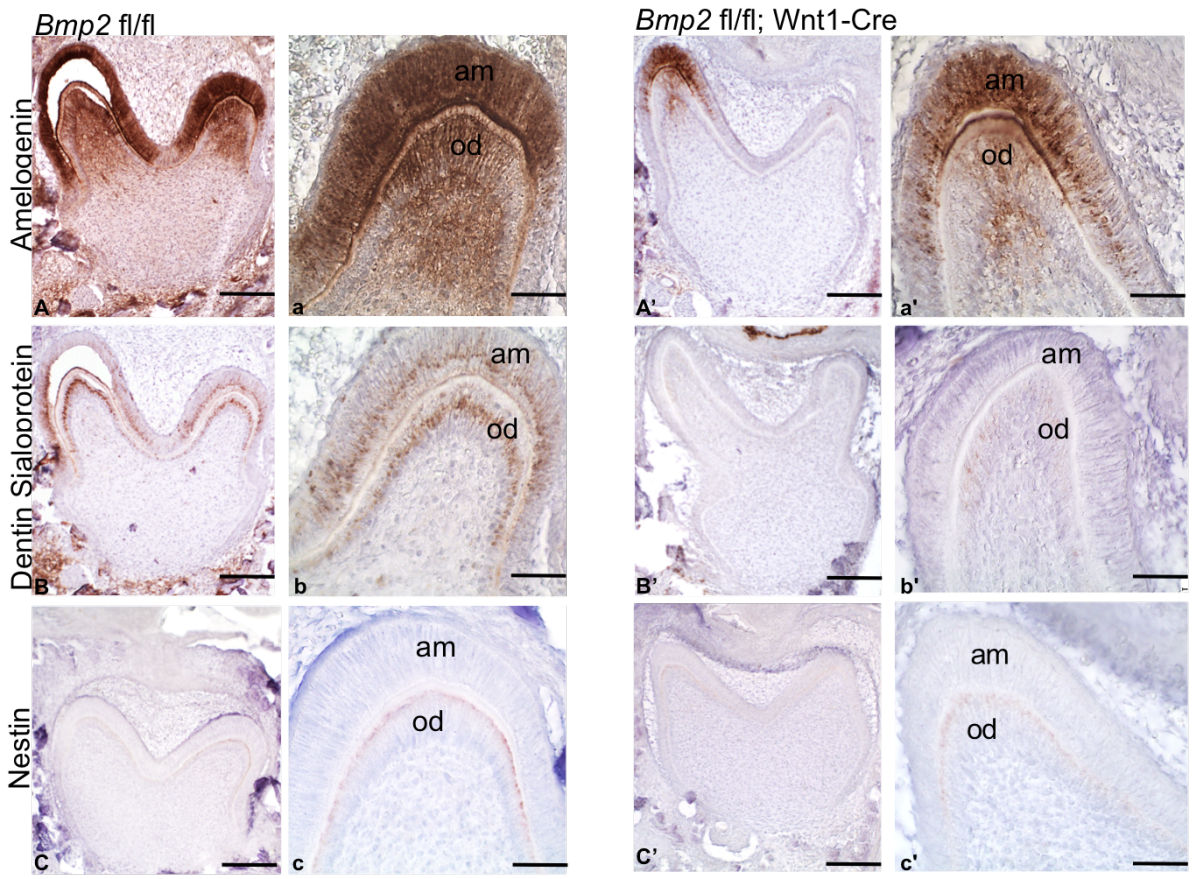


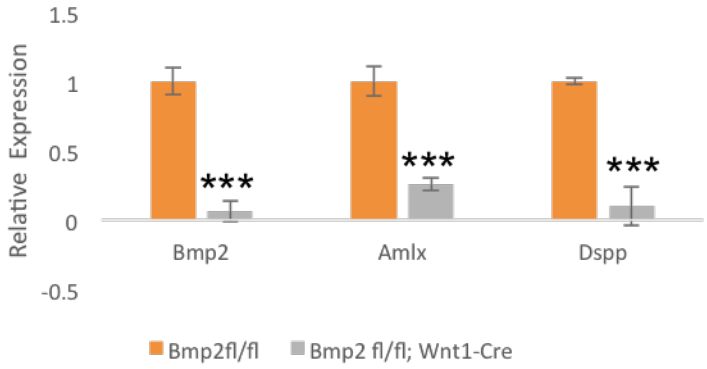
Figure 6.14- Proliferation and apoptosis at P0. (A, A”) Immunohistochemistry for Ki67 (brown) in mutant (A”) and control (A) to assess proliferation. Sections were counterstained with hematoxylin. Note areas surrounding tooth positive for Ki67 (arrows). (B,B”) Immunohistochemistry for caspase (purple) to assess apoptosis between mutant (B”) and control (B). (b,b`) Higher magnification images of B and B`showing apoptosis in ameloblasts and odontoblast cell layer. Scale bar A,A`,B,B`- 100µm ; b,b`-20µm.

6.3.6 Altered expression of dentin matrix proteins in *Bmp2* deficient tooth mesenchyme

To assess specific defects to odontoblasts or tooth minearlization, we performed immunohistochemistry for odontoblast and enamel markers in P0 mandibular molars. Differences in Dentinsialoprotein (Dsp) a product of odontoblasts secreted into the dentin layer were apparent in mutants (Figure 6.15 b,b`). The preodontoblast marker Nestin was also reduced in mutants (Figure 6.15 c,c`). Interestingly, the ameloblast marker amelogenin was also significantly reduced (Figure 6.15 a, a`), indicating that both dentin and enamel production were affected. These findings were confirmed by quantitative RT-PCR (qPCR) showing reduced expression levels of all three genes (Figure 6.15 II). These results show that *Bmp2* deletion in mesenchyme affects both the dentin as well as the enamel matrix (low amelogenin expression), indicating a possible epithelial-mesenchymal interaction.



I.



II.

Figure 6.15: Protein and Gene expression at P0. I. Immunohistochemistry for Amelogenin (Amlx), Dentin Sialoprotein (Dsp), and Nestin reveal a delay in odontoblast and ameloblast maturation in mutant (A'-C') as compared to the control (A-C). Dsp and Nestin marks odontoblasts and Amelogenin marks ameloblasts. (a-c and a'-c') magnification showing staining in ameloblast and odontoblast cells of control (a-c) and mutant (a'-c'). Note reduced expression of Dsp. II. Quantitative RT-PCR on cDNA from total tooth RNA shows significant *Bmp2* reduction, along with reduced Amelogenin and Dspp (***P<0.0001) (n=5). Abbreviation: am-ameloblasts, od-odontoblasts. Scale bar (A-C, A'-C'): 100µm (a-c, a'-c'): 50µm.

pSmad 1/5/8 serves as a read-out for the Bmp signaling pathway. To assess sites of changes to Bmp signaling in the absence of *Bmp2* during tooth development, pSmad 1/5/8 immunohistochemistry was performed. We found overall reduction of pSmad1/5/8 signal both in mesenchymal odontoblasts and epithelial ameloblasts (Figure 6.16 a, a`) and Hertwig's epithelial root sheet (HERS) cells (Figure 6.16 b, b`, c, c`). This shows that deletion of *Bmp2* from neural crest mesenchyme causes overall reduction in Bmp signaling in both ameloblasts and odontoblasts indicative of changes in epithelial-mesenchymal interactions.

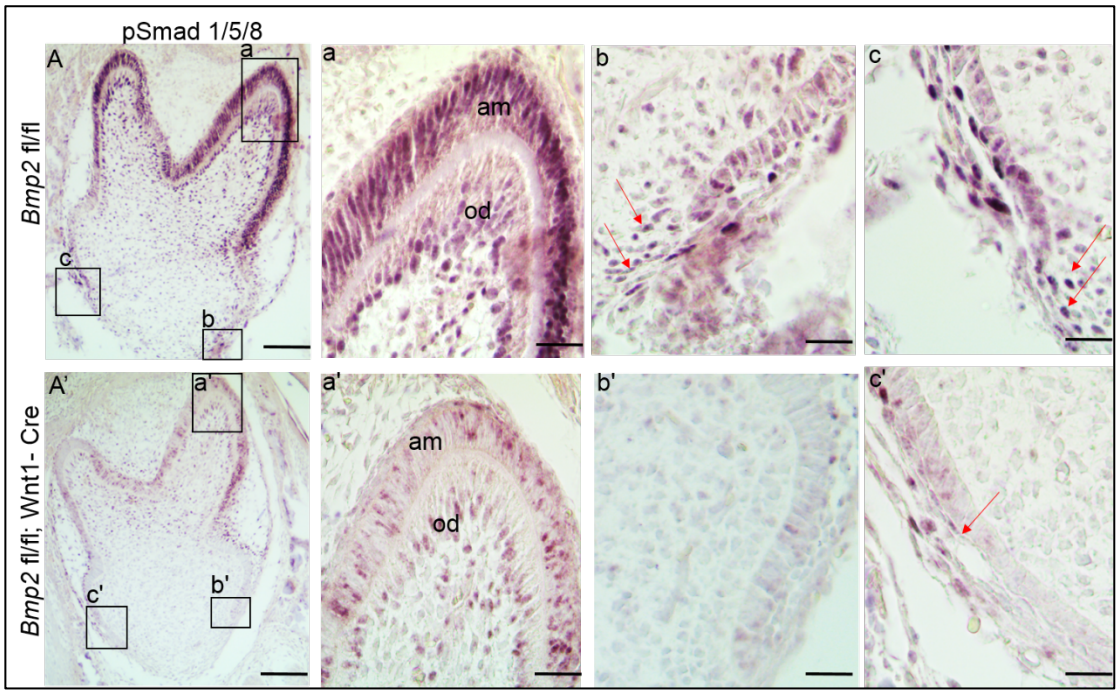
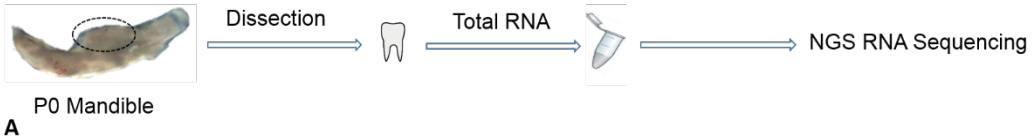


Figure 6.16: Bmp signaling in both epithelial and mesenchymal tooth derivatives. (A, A') Immunohistochemistry for phosphorylated Smad1/5/8 shows active Bmp signaling in tooth in control (A) and mutant P0 tooth (A'). (a, a') magnified image shows reduced expression of pSmad1/5/8 in both ameloblast (am) and odontoblast (od) cells. (b,b' and c,c') magnified image showing less or no staining in HERS (arrows). Scale bar (A,A'): 100µm; (a-c, a'-c'): 50µm.

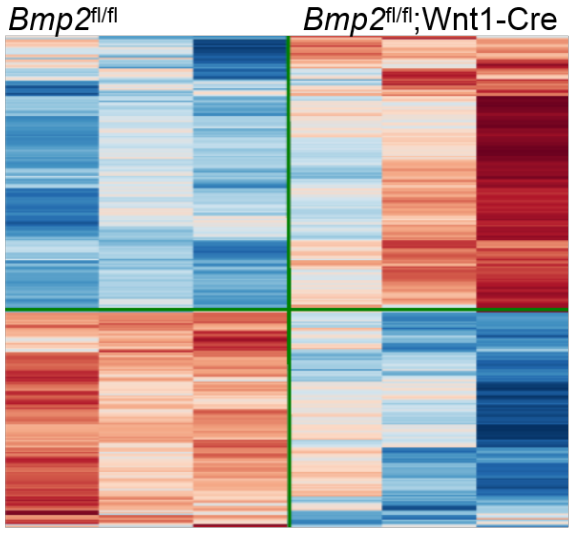
6.3.7 RNA-seq analysis of *Bmp2* deficient tooth mesenchyme at P0

To understand how *Bmp2* deletion in tooth mesenchyme affects global gene expression we performed RNA-seq analysis on isolated P0 molars. Mutant and control molars from lower

mandibles of P0 were individually dissected and total RNA was extracted from pooled molars from one embryo (Figure 6.18A). The RNA-seq was performed by the Functional Genomics Centre Zurich (FGCZ) inclusive of quality control, initial bioinformatics, as well as statistical analysis of the RNA-seq data. Quality control showed slight degradation of some of the RNA samples possibly a consequence of the lengthy dissection procedure. Whereas initial analysis did not give a clear separation by unsupervised hierarchical clustering between mutant and control samples, statistical correction to take differences in RNA size in consideration (performed by FGCZ) resulted in a heat map with good separation between mutant and control samples (Figure 6.12 B). Enrichment analysis report by gene network and gene ontology was performed (Table 6.1 and Table 6.2). This revealed differential expression of 15% of genes associated with bone and cartilage development, 20% genes in Wnt_TGF_cytoskeleton remodeling, as well as 22% genes in skeletal muscle development. Selected genes from these different pathways were validated using qPCR.



A



B

Figure 6.18- RNA seq data for isolated mandibular molars from P0 pups. (A) Illustration showing sample collection and RNA isolation from P0 mandibular molars. (B) Heat plot of gene expression in $Bmp2^{fl/fl}$; Wnt1-Cre mutant vs littermate controls ($Bmp2^{fl/fl}$)

Enrichment analysis	***P*-value**
Development_signaling pathways in bone and cartilage development	2.90E-06
Oxidative phosphorylation	2.80E-06
Transcription_Role of VDR in regulation of genes involved in osteoporosis	6.60E-05
Cytoskeletal remodeling_TGF_WNTs	2.30E-04
Cell adhesion_ECM remodeling	3.09E-04
LRRK2 in neurons in Parkinsons disease	5.40E-04
Cell adhesion, Chemokines and adhesion	9.90E-04
Ubiquinone metabolism	1.70E-04

Table 6.1. Enrichment analysis showing altered biological processes**.**

Enrichment analysis by GO process	***P*-value**
Single organism developmental process	2.60E-10
Response to O_2 containing compound	4.05E-10
Establishment of protein localization to the membrane	8.30E-10
Organ development	6.74E-10
Response to inorganic substance	7.30E-09
Response to organic substance	1.16E-09
Response to lipid	5.37E-09
Anatomical structure development	1.70E-08

Table 6.2. Enrichment by GO procesess

6.3.8 Validation of RNA-seq data using RT-PCR analysis

As isolation of P0 molars was difficult and time consuming resulting in compromised RNA quality, we resorted to RNA extraction from molars inclusive of part of the mandible for validation purposes. We reasoned that better quality material for qPCR analysis would outweigh the disadvantage of working with a lesser pure material.

RNA was thus extracted from mandibular molars with alveolar bone attached followed by cDNA synthesis and qPCR. We tested for differences in the Bmp pathway, the Wnt pathway, genes associated with enamel and dentin differentiation in alignment with results from the enrichment analysis. We confirmed loss of *Bmp2,* found statistical significant upregulation of *Bmp3 and Bmp4*, as well as downregulation of *Bmp5* and *Bmp6*. The Bmp and Activin antagonist *Follistatin* was significantly down regulated (Figure 6.19B). This indicated that loss of *Bmp2* led to a more general deregulation of the Bmp pathway in P0 molars. With respect to the Wnt pathway most of the Wnt genes investigated were down regulated, whereas the Wnt inhibitors *Sost* and *Dkk1* were up regulated (Figure 6.19 C). These results indicate that in addition Wnt signaling is strongly affected by loss of Bmp2.

During early stages of tooth development, *Bmp2* has been shown to be involved in epithelial-mesenchymal interactions, whereby epithelial *Bmp2* is thought to regulate genes such as *Pax9* and *Msx1/2* in the mesenchyme. To address whether mesenchymal Bmp2 participates in a mesenchymal-epithelial crosstalk, we tested changes to gene expression in isolated tooth epithelium and mesenchyme from P0 molars. This revealed that several genes associated with enamel formation, such as, *Amelx*, *Ameloblastin (Ab), Mmp20* were downregulated. In contrast, several oral epithelial genes such as *Dccp1, Dcpp2, Dcpp3, Aqp5* and *K14* showed no difference (Figure 6.20B), with the exception of *Muc19*, a mucin gene important in formation of gel like properties of mucin (Figure 6.20B).

These findings support a mesenchymal-epithelial interaction, whereby odontoblast-derived *Bmp2* provides a critical differentiation signal for ameloblasts differentiation to the mineralization

stage.

In addition, we found that the dentin matrix protein-encoding genes *Dspp, Dmp1*, the *Bmp2* downstream gene *Runx2*, as well as several bone related genes [*Osteocalcin (Oc), osteoprotegrin (Opg), osteonectin (On)]* were downregulated in the mesenchyme (Figure 6.20 C). In contrast, *Col1a1, Nestin* and *Bsp* did not show any differences. This indicates that loss of mesenchymal *Bmp2* affected some but not all functional aspects of odontoblasts.

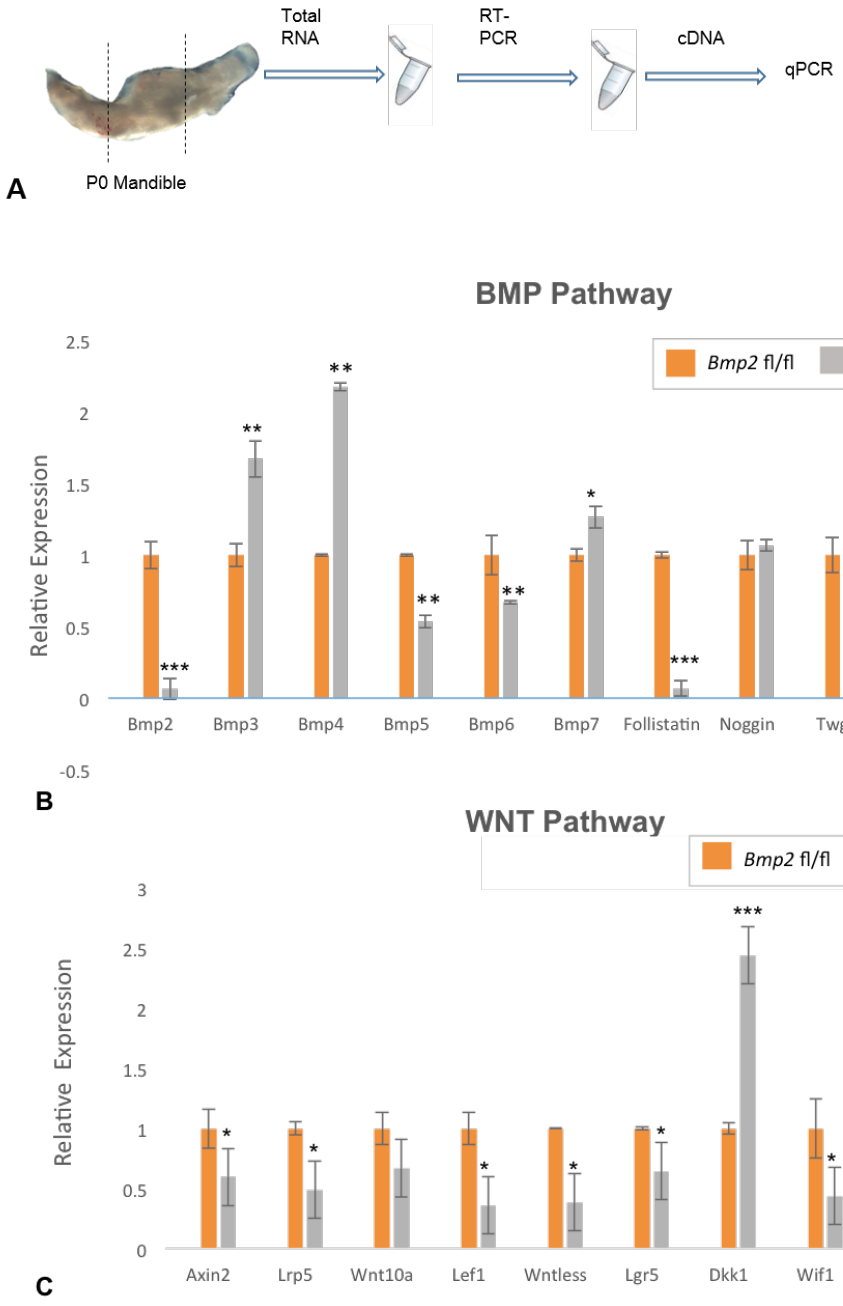


Figure 6.19: Validation of qPCR data of selective genes. (A) Schematic showing RNA isolation. (B) De-regulation in mutant and control. (C) De-regulation of genes in **Wnt pathway**. Statistically significant p values are marked with an asterisk (*P<0.01,**P<0.001,***P<0.0001) (n=2).

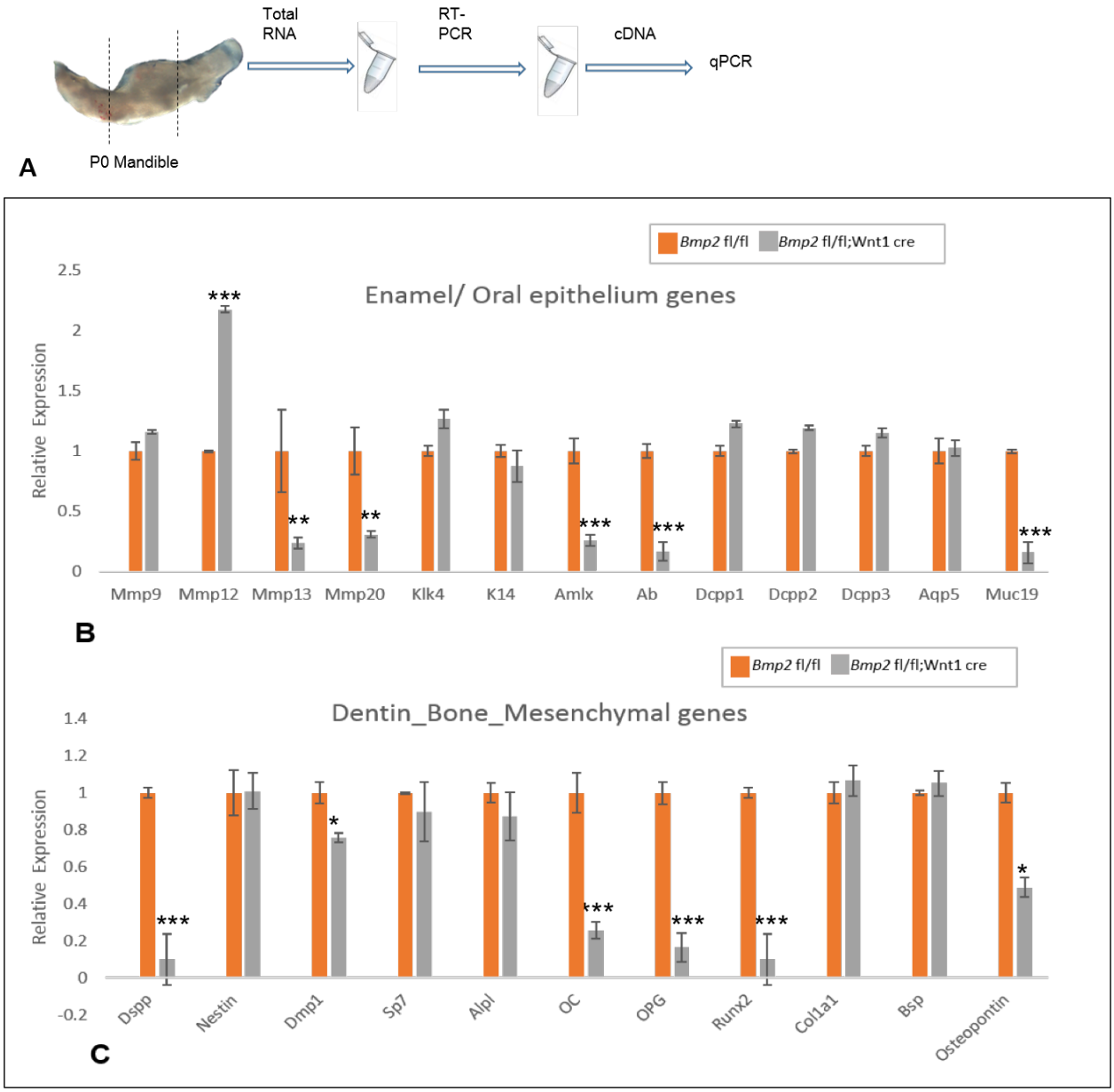


Figure 6.20: Validation of qPCR data of selective genes. (A) Schematic showing RNA isolation. (B,C) Differential expression of **epithelial and mesenchymal genes**. Statistically significant p values are marked with an asterisk (*P<0.01, **P<0.001,***P<0.0001) (n=3).

6.3.9 Transcription factors in *Bmp2* deficient tooth

Next we validated some transcription factors downregulated in the RNA-seq analysis (*Pax9, Msx1, Tbx1*) and tested some transcription factors with known role in tooth development for example *Pitx2, Barx1, Sox2, Sox9*. Significant down regulation of *Pax9, Msx1, Tbx1 and Sox9* was noted whereas *Pitx2* was not affected (Figure 6.21B). *Pax9* is expressed in the mesenchyme during early tooth development and it has an important role for maintenance of mesenchymal *Bmp4*, which is critical for early tooth morphogenesis (Chen et al., 1996) (Peters et al., 1998) (Jia et al., 2013). Mutations in PAX9 cause selective tooth agenesis in humans (Mostowska et al 2003). Immunohistochemical analysis revealed the surprising expression of *Pax9* prominently in the epithelial layer (ameloblasts), which was lost in *Bmp2* mutants (Figure 6.21 C). These results indicate that mesenchyme-derived *Bmp2* might regulate ameloblast differentiation via Pax9 -> Bmp4 further supporting a role for *Bmp2* in mesenchymal-epithelial interaction.

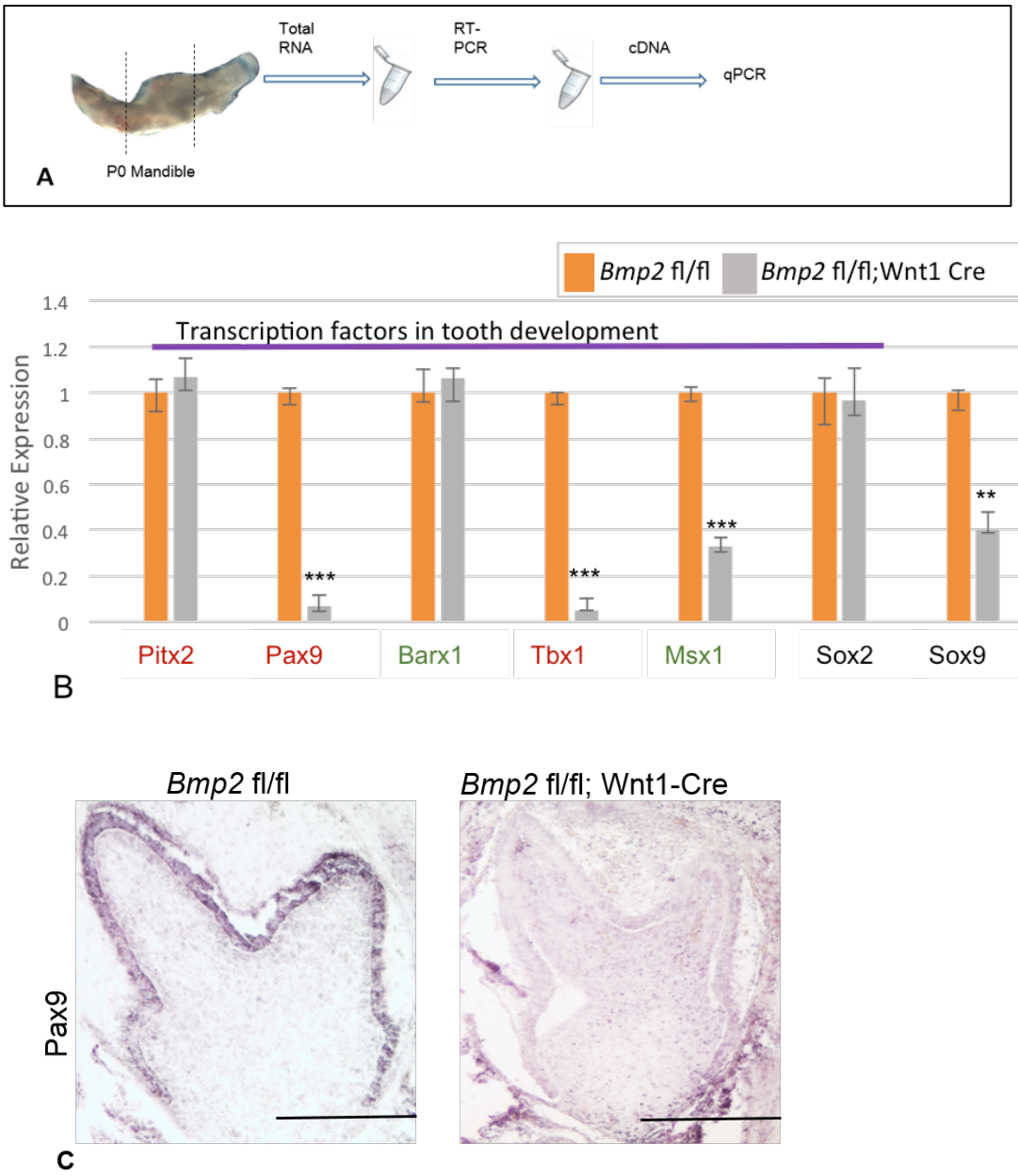
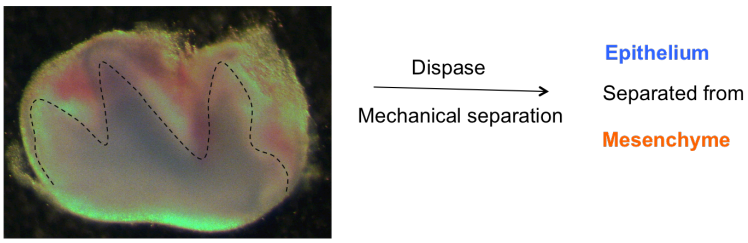


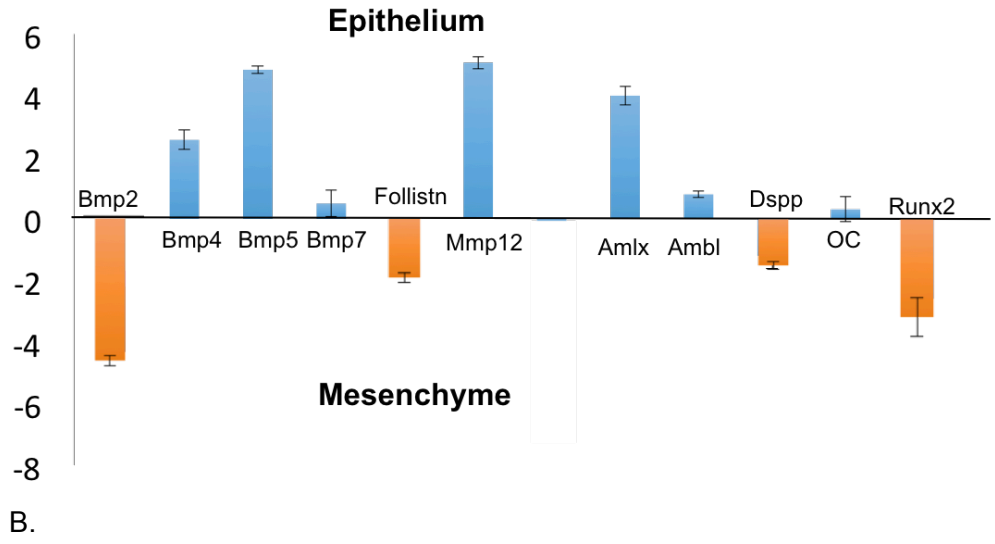
Figure 6.21: Validation of qPCR data of selective genes. (A) Schematic showing RNA isolation. (B) Differential expression of **transcription factors in tooth development**. (C) Immunohistochemistry showing Pax9 expression. Scale bar (C) 100µm. Statistically significant p values are marked with an asterisk (*P<0.01,**P<0.001,***P<0.0001) (n=2).

6.3.10 Separation of Mesenchyme and Epithelium of P0 molars

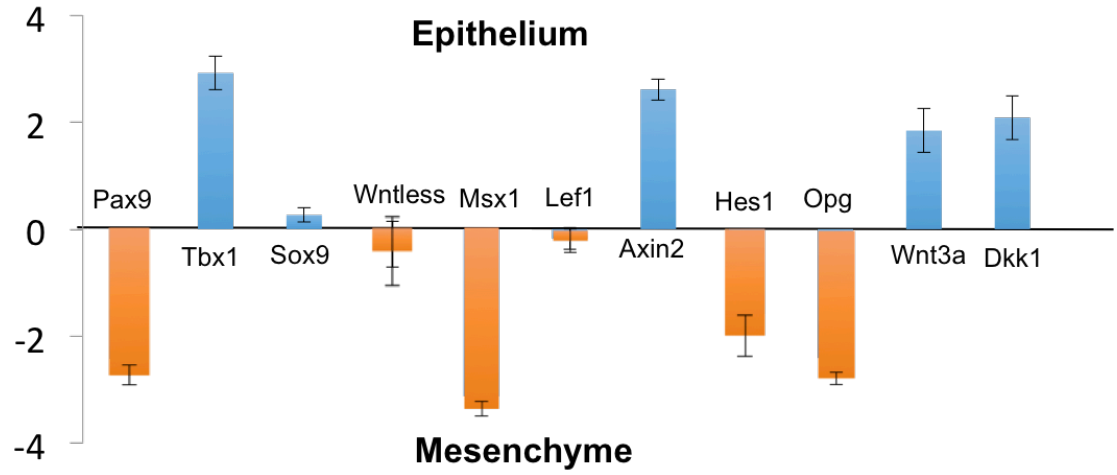
To further study *Bmp2* function in E-M and M-E interactions we carefully dissected P0 molars and separated epithelium from mesenchyme. For this, isolated teeth were incubated in Dispase for 20 mins and mesenchyme was separated from the epithelium mechanically (Figure 6.22 A). Tissue specific expression within mesenchyme or epithelial genes was calculated using the ΔCq values obtained from the qPCR analysis. This method is an arbitrary calculation performed to understand which genes have a higher expression in which tissue (epithelium or mesenchyme). *Bmp2, Follistatin, Runx2* were expressed more in mesenchyme whereas *Bmp4, Bmp5, Mmp12, Amlx*, *Ameloblastin (Ambl)* were expressed more in epithelium*. Bmp7 and osteocalcin (OC)* were expressed in almost equal amounts in both epithelium and mesenchyme (Figure 6.22B). Transcription factor *Pax9, Msx1* was more in the mesenchyme whereas *Tbx1* and *Sox9* were in the epithelium. *Osteoprotegrin (OPG)* was also expressed more in the mesenchyme. *Axin2*, *Wnt3a* and *Dkk1* were expressed more in the epithelium. *Wntless, Hes1* and *Lef1* were slightly higher in mesenchyme (Figure 6.22C).



A.



B.



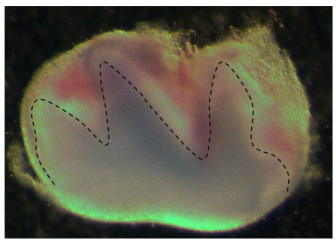
C.

Figure 6.22 Expression of epithelial and mesenchyme genes in control tooth (Values calculated using ΔCq of control mesenchyme- ΔCq of control epithelium)

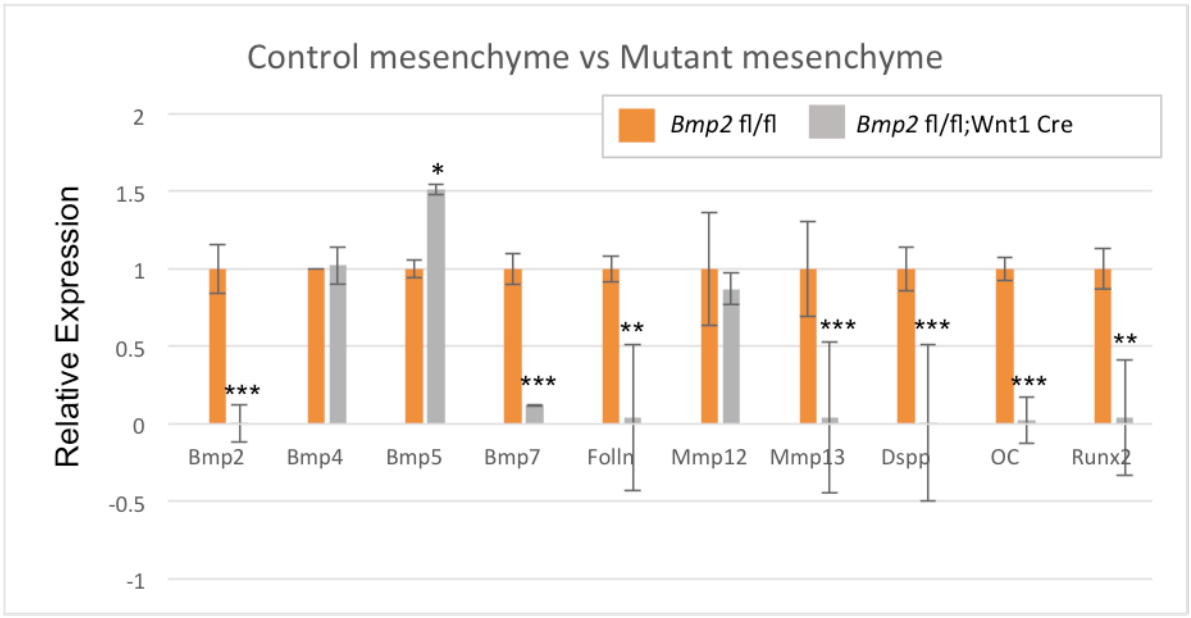
6.3.11 *Bmp2* is an important signal for reciprocal E-M interaction during early mineralization

To further study *Bmp2* function in E-M and M-E interactions we compared separated epithelium from mesenchyme from mutant and control teeth. qPCR analysis on selected genes was performed. *Bmp2* was downregulated as expected in mutant mesenchyme along with *Follistatin, Mmp13, Dspp, Runx2* and *OC,* all the genes found to be downregulated in RNA-seq data (Figure 6.23 B). Analysis of mutant epithelium revealed upregulation of *Bmp4*, *Bmp7, Mmp13* and complete loss of Ameloblastin (Figure 6.23 C). This suggests that up-regulation of Bmp4 is not a compensation mechanism but a consequence of loss of Bmp2- signaling to epithelial cells. Interestingly, *Pax9* was upregulated in contrast to reduced Pax9 protein expression in mutant teeth. It is thus possible that Pax9 is subject to a translational control dependent on Bmp2 signaling. Overall, these results further corroborate the importance of *Bmp2* for reciprocal epithelial-mesenchymal interactions at the mineralization stage of tooth formation.

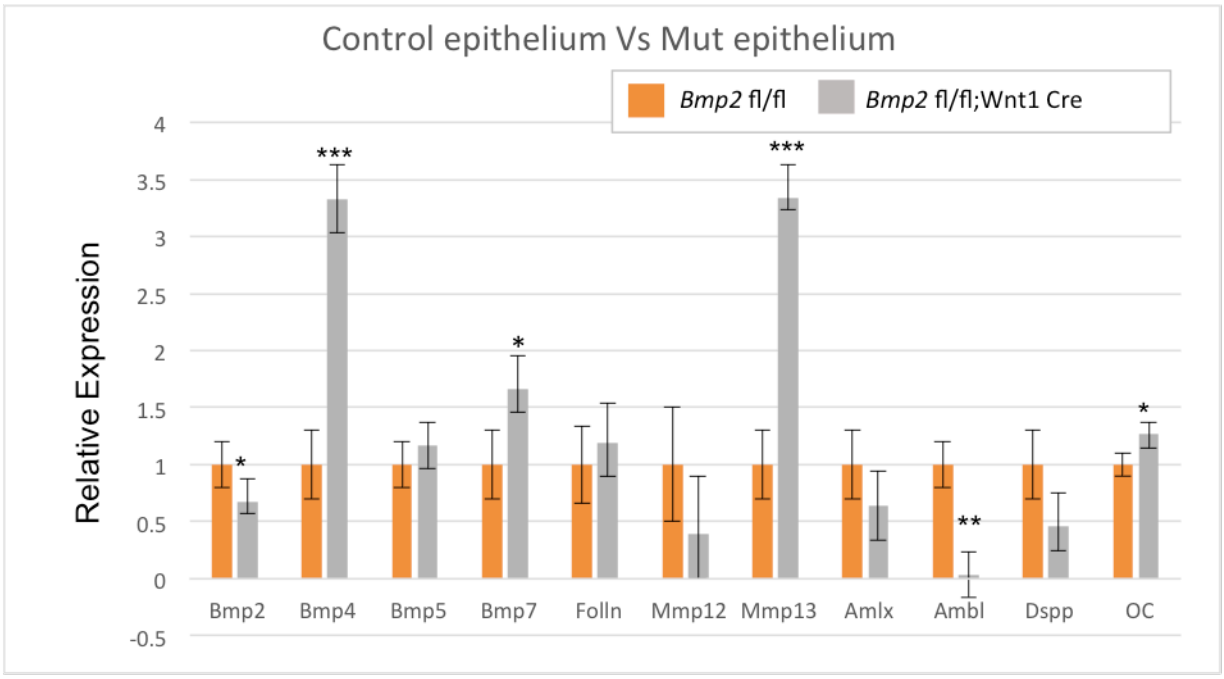
Various Wnt pathway genes were also downregulated in the mesenchyme: *Hes1, Axin2, and Wnt3a* (Figure 6.23 D, E). Also an overall decrease in Wnt signaling was observed as shown by reduced β-catenin staining (Figure 6.23 F). *Dkk1* was upregulated in the epithelium (Figure 6.23 D, E). This indicates that *Bmp2* also plays a role in Wnt-mediated odontoblast formation.


Dispase
Mechanical separation
Epithelium
Separated from
Mesenchyme

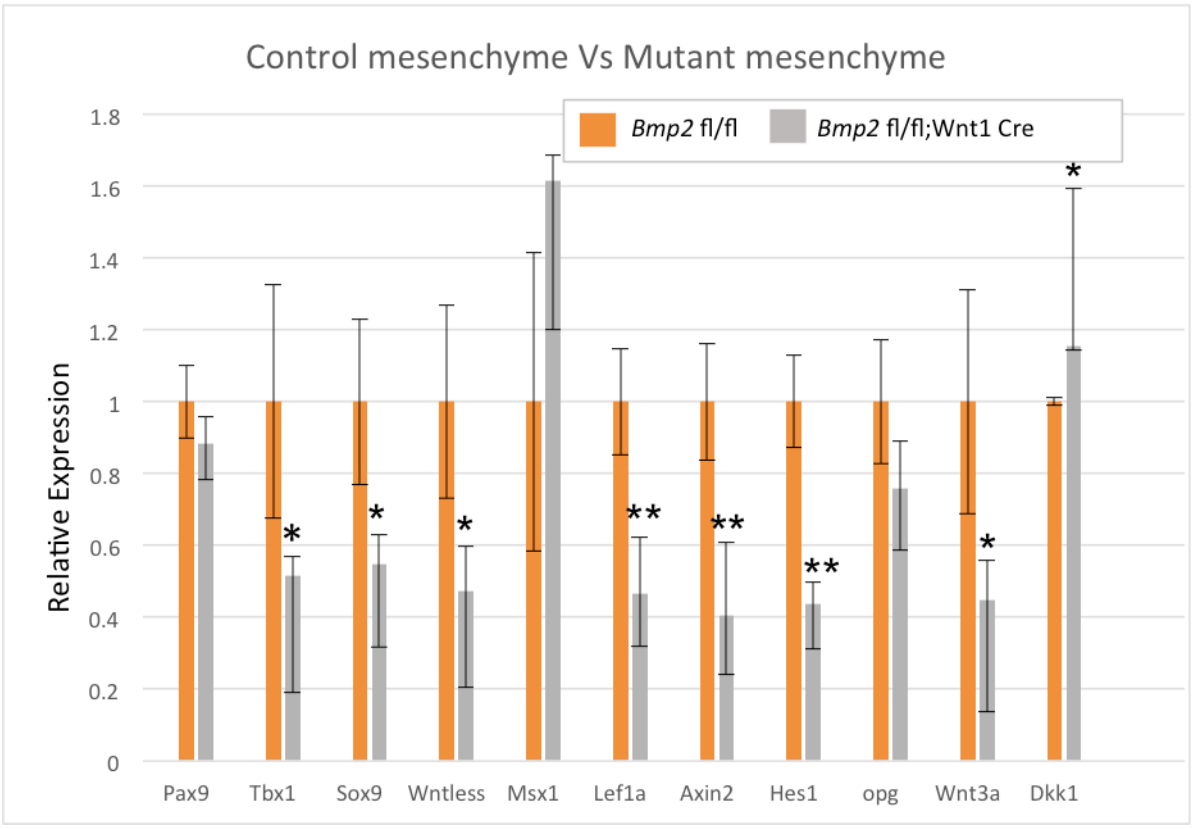
A.


Control mesenchyme vs Mutant mesenchyme
Bmp2 fl/fl
Bmp2 fl/fl;Wnt1 Cre
Relative Expression
2
1.5
1
0.5
0
-0.5
-1
Bmp2
Bmp4
Bmp5
Bmp7
Folln
Mmp12
Mmp13
Dspp
OC
Runx2

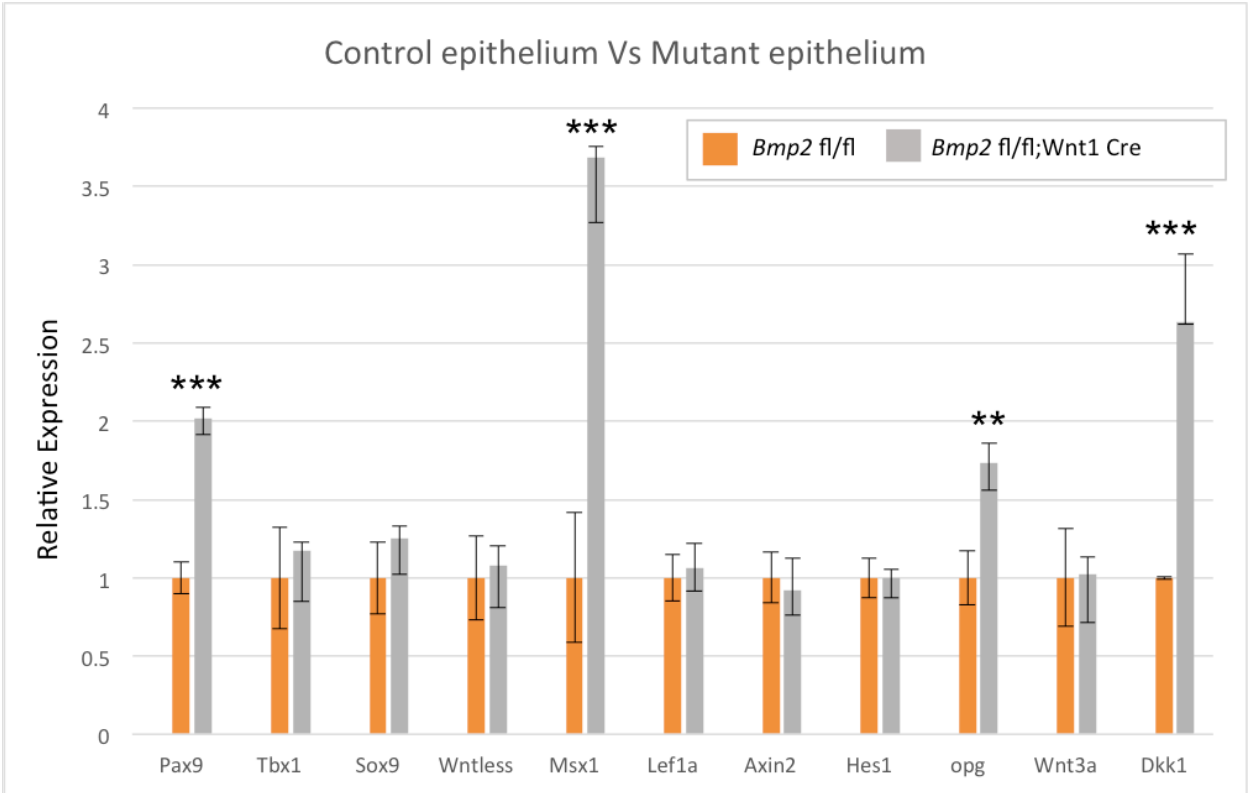
B.


Control epithelium Vs Mut epithelium
Bmp2 fl/fl
Bmp2 fl/fl;Wnt1 Cre
Relative Expression
4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
Bmp2
Bmp4
Bmp5
Bmp7
Folln
Mmp12
Mmp13
Amlx
Ambl
Dspp
OC

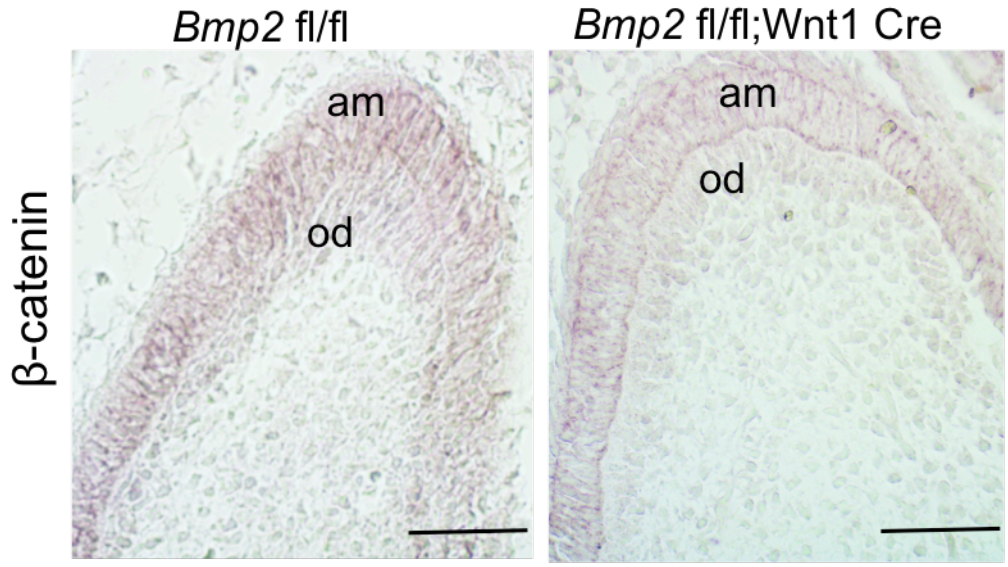
C.


Control mesenchyme Vs Mutant mesenchyme
Bmp2 fl/fl
Bmp2 fl/fl;Wnt1 Cre
Relative Expression
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Pax9
Tbx1
Sox9
Wntless
Msx1
Lef1a
Axin2
Hes1
opg
Wnt3a
Dkk1

D.


Control epithelium Vs Mutant epithelium
Bmp2 fl/fl
Bmp2 fl/fl;Wnt1 Cre
Relative Expression
4
3.5
3
2.5
2
1.5
1
0.5
0
Pax9
Tbx1
Sox9
Wntless
Msx1
Lef1a
Axin2
Hes1
opg
Wnt3a
Dkk1

E.



F.

Figure 6.23- RT-PCR from separated P0 molar epithelium and mesenchyme. (A-E) Statistically significant p values are marked with an asterisk (*P<0.01,**P<0.001,***P<0.0001) (n=2). (F) β-catenin immunohistochemistry of mutant (right) and control (left) showing an over all decrease in the mutant odontoblasts (right) and also in ameloblasts (right).

Part III: *Bmp2* deficient tooth germ mesenchyme grows into an adult tooth upon ectopic Kidney Transplantation

6.3.12 *Bmp2* deficient tooth germ forms adult tooth with dentin defects.

As our *Bmp2*fl/fl; Wnt1-Cre mouse model is only viable up to P0 stage of postnatal development, this limits our study to address *Bmp2* deficiency in adult tooth mesenchyme. To overcome this problem we used the kidney capsule transplantation (KCT) method to grow *Bmp2*-deficient tooth germs *in vivo*. For this, E13.5-E14.5 tooth germs were dissected carefully separating molar and incisors. Genotyping was performed to identify mutant and control tooth germs. Subsequently, selected tooth germs were transplanted under the peritoneum layer of the kidney (kidney capsule) of adult recipient mice. Kidneys were harvested 4 weeks after transplantation (Figure 6.24 A-D), a time point when mature teeth have formed.

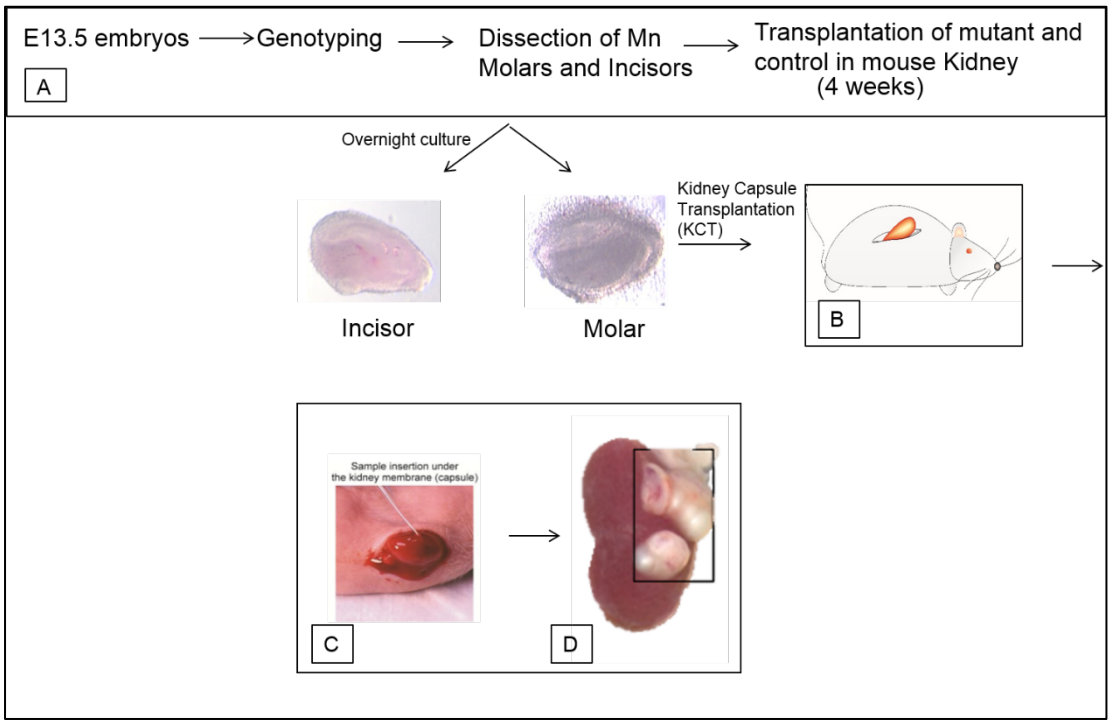
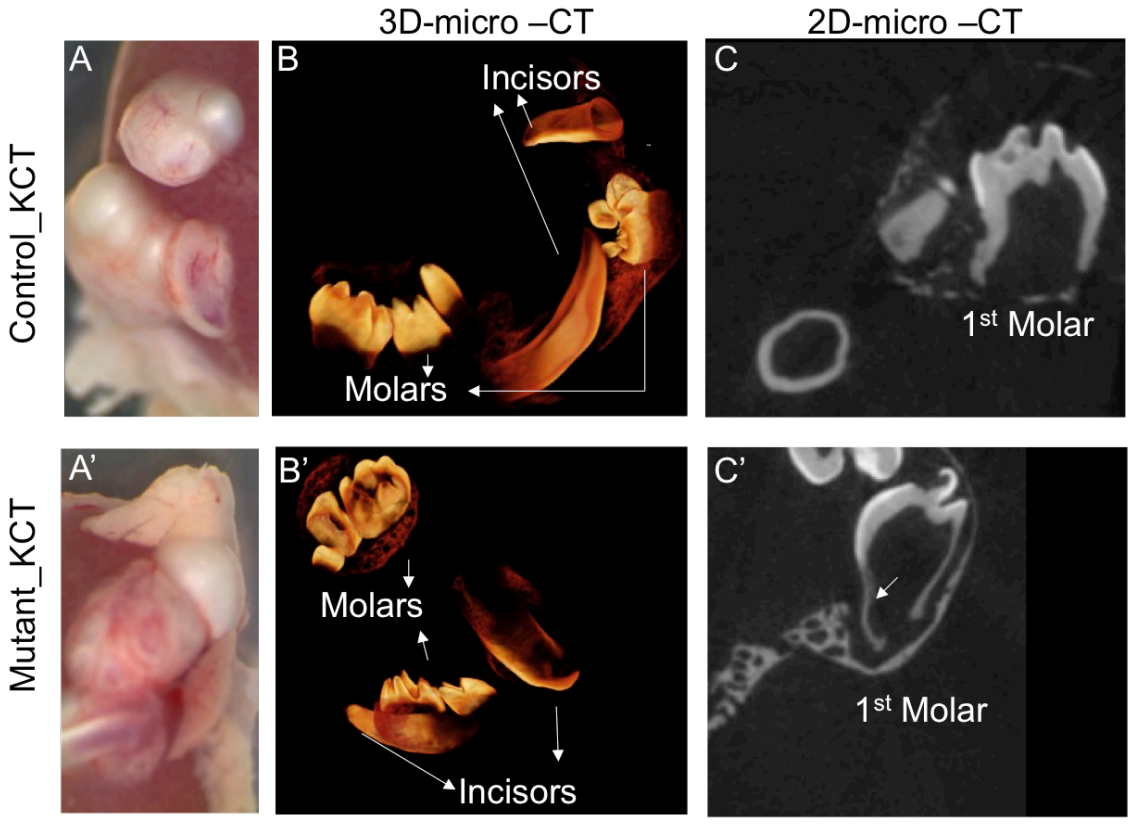
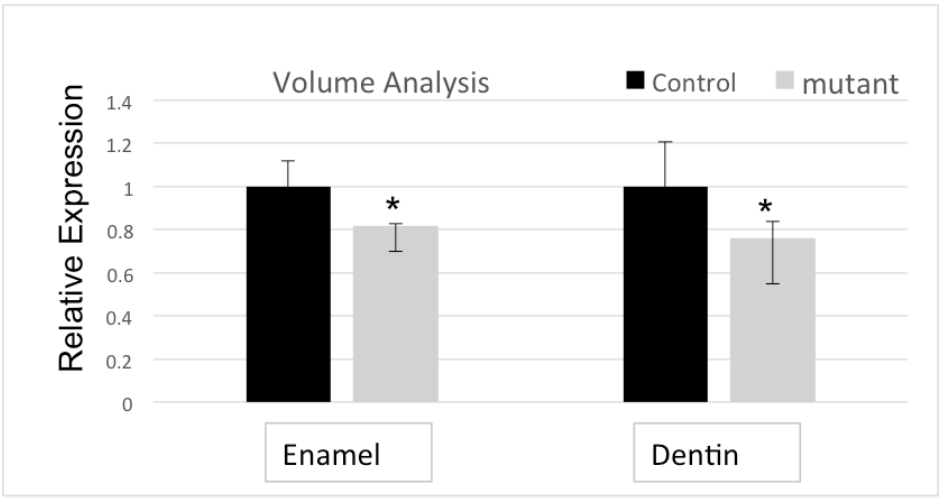


Figure 6.24: Schematic of maturation of teeth by transplantation of tooth germs under the kidney capsule: (A) Schematic showing the different steps involved in harvesting tooth germs and transplantation of tooth germs under kidney capsule. (B) Illustration showing Kidney capsule transplantation (KCT) of molar and incisor tooth germ in mouse. (C, D) Formation of adult tooth on kidneys after 4 weeks following transplantation.

µCT analysis of the teeth grown on the kidneys revealed that both molars and incisors were formed from mutant tooth germs, with three molars and one incisor from each quadrant of the mice jaw. Thus, loss of *Bmp2* does not lead to an anomaly in tooth number (Figure 6.25 A, A`,B, B`). 2D-µCT representations revealed morphological differences apparent as thinner dentin towards the root area with irregular dentin formation along the pulp-dentin junction (Figure 6.25 C, C`). Volumetric analysis showed a slight but significant decrease in the volumes of both enamel and dentin (Figure 6.25 D, D`). To further corroborate changes to dentin structure we performed scanning electron microscopy (SEM). This revealed irregular dentin formation and ectopic bone like structures in the pulp area (Figure 6.26 A, B, C, D). This type of ectopic structure is commonly referred to as osteo-dentin or interglobular dentin due to its similarity to both bone and dentin structures (Goga et al., 2008). Little is known about the etiology of osteodentin formation.





D

Figure 6.25: In vivo growth of Bmp2 deficient (A') and control tooth germs (A) under host kidney capsule dissected 4 weeks after transplantation. (B, B') 3D-μCT image of KCT grown teeth showing mineralized adult teeth (including both molars and incisors). (C, C') 2D cross section of microCT recording reveals that these irregularities are calcified (electron dense) (arrow) indicative of pulp obliteration/formation of pulp stones. (D) Histogram showing differences in volume of dentin and enamel. Note significant decrease in both dentin and enamel volume (*P<0.01)(n=3).

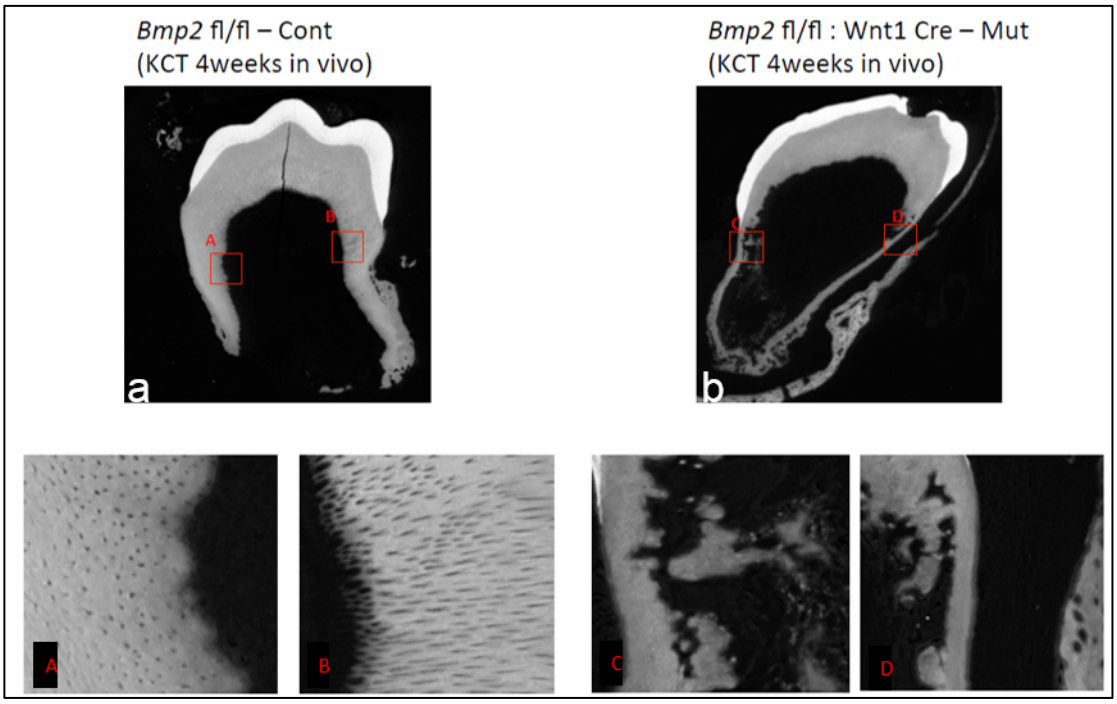


Figure 6.26: (a,b) **SEM analysis** confirms the irregular dentin formation along with ectopic mineralization in the pulp. (A, B, C, D) magnified image of (a,b) revealing differences in dentin structure formation in mutant (C,D) and control (A,B).

6.3.13 Immunohistochemical analysis of Bmp2 deficient adult tooth

Histological analysis confirmed the formation of osteodentin in the pulp as well as the irregular dentin-pulp border. Osteodentin appeared always attached with dentin and no isolated osteodentin was found in the pulp. The osteodentin phenotype was variable and ranged from mild to severe, with some mutant tooth covering 2/3rd of the surface area of the pulp. A representative example is shown in Figure 6.27 A, A, B, B` with osteodentin formation. Osteodentin consisted of bone like cells or odontoblast like cells trapped in irregular dentin (Figure 6.27 B, B`-arrows). pSmad1/5/8 staining revealed changes to Bmp signaling in pulp and osteodentin. Active Bmp signaling was observed in the osteodentin structures (Figure 6.27 C,C`). Overall, Bmp signaling in root and periodontal ligament along with alveolar bone was decreased in *Bmp2* mutant tooth (Figure 6.28). This shows that *Bmp2* is important for fine regulation of pulp structures in adult teeth.

To further characterize the observed osteodentin immunohistochemistry was performed. Altered expression of Bone sialoprotein (Bsp), Nestin and Dentin sialoprotein (Dsp) was observed (Figure 6.29). Dsp was found in the osteodentin structures supporting loss of cellular organization within the dentin structure. Bone sialoprotein was also found in osteodentin structures.

In summary we have shown that *Bmp2* is an important factor in epithelial mesenchymal interaction at early mineralization stages. *Bmp2* is important for formation of dentin and odontoblast differentiation via Wnt signaling and Pax9. And *Bmp2* is required for homeostasis of the pulp tissue in adult tooth.

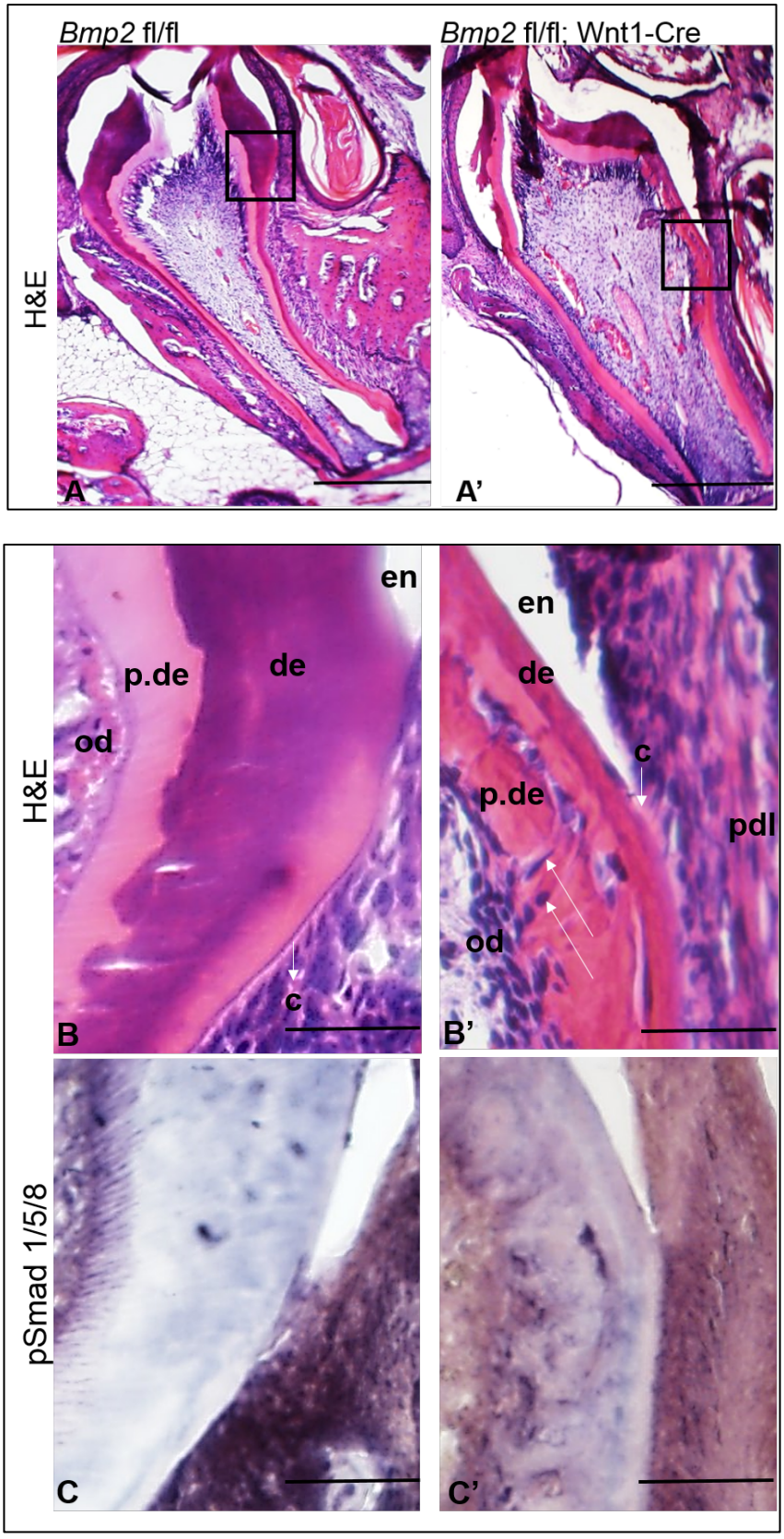


Figure 6.27: Deletion of Bmp2 in the dental pulp leads to pulp obliteration, irregular cementum and asymmetric dentin dysplasia : (A, A') Hematoxylin/Eosin stained Paraffin sections of mutant (A') and control (A) KCT tooth reveal altered appearance of the pulp tissue in Bmp2-deficient teeth.

(B, B') magnification of (A, A') showing irregular borders between pulp tissue, pre-dentin (light pink), and mature dentin (dark pink) and formation of inclusions (arrows). Cementum structure is also disrupted (arrow) (C,C') Active Bmp signaling in the dentin structure, cementum, periodontal ligament as revealed by pSmad1/5/8 staining. Note also staining in cell inclusions. Abbreviations: en-enamel; de-dentin, p.de-predentin, od-odontoblasts, c-cementum, pdl-periodontal ligament. Scale bar (A,A'): 200µm; (B,B' and C,C'): 50µm.

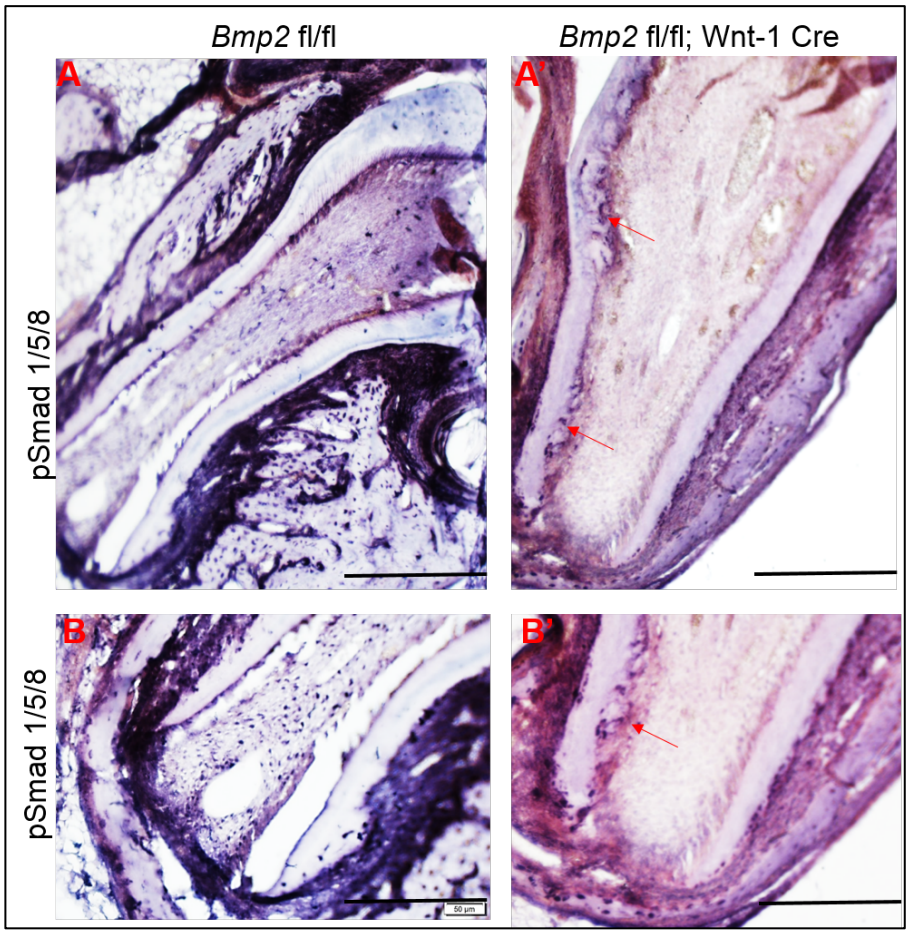


Figure 6.28: Overall reduction of active Bmp signaling in root, alveolar bone and periodontal ligaments as shown by pSmad staining. (A, A') pSmad 1/5/8 of control (A) and mutant (A') KCT tooth. (B, B') magnified images of (A, A') showing active Bmp signaling in ectopic mineralized structures of the tooth (arrows). Note that obliterated tissue contained pSmad1/5/8 positive nodules indicating that loss of Bmp2 might be lead to altered/ectopic Bmp signaling (other than by Bmp2) at these sites.Scale bar (A, A'): 100µm (B,B') 50µm.

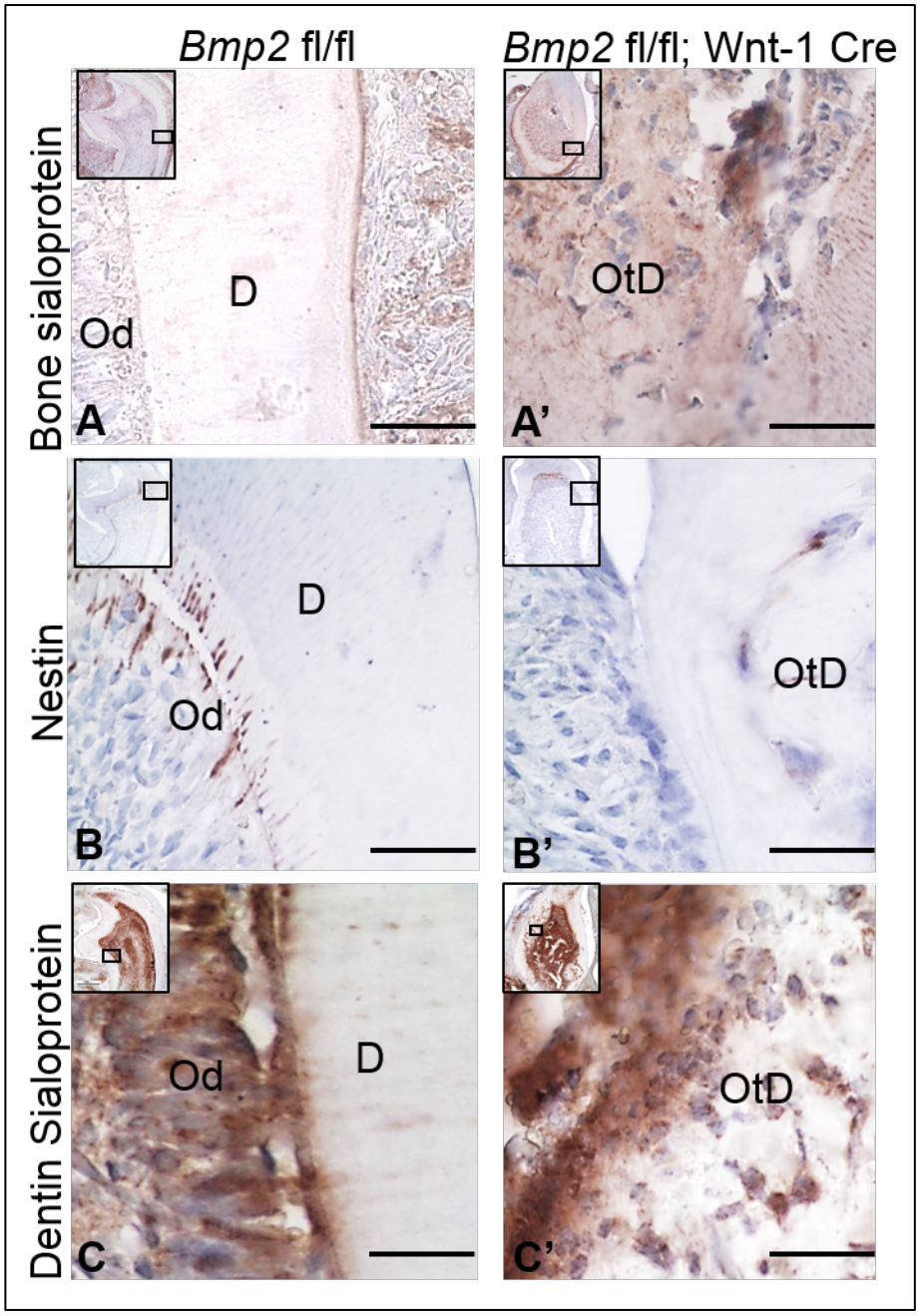


Figure 6.29: Immunohistochemical characterization of Bmp2-deficient pulp. (A, A') Bone sialoprotein staining in ectopic mineralized areas of the mutant KCT tooth (A') reveals an important characteristic of these cell types (therefore will be referred as Osteo-dentin (OtD)). (B, B') Nestin remained reduced and was lost at the apical ends at sites where tissue disorganization was apparent in mutant (B') (C, C') Staining for Dsp revealed partial loss of pulp organization and ectopic deposition of Dsp within the primary dentin of mutant teeth. Abbreviations: OtD- OsteoDentin, D-Dentin. Scale Bar (A,B,C): 100μm; (A',B',C): 50μm.

7. Discussion

7.1 Epithelial *Bmp2* is important for enamel formation in mouse incisors

In the first part of this study we have shown that conditional deletion of *Bmp2* in epithelium caused delayed mineralization in incisors. Histochemical analysis revealed disrupted Stratum intermedium (SI) epithelial cell layer, which in turn might affect ameloblast differentiation and enamel formation, as an intact SI layer has been shown to be critical for these processes (Wise and Fan, 1989) (Kawano et al., 2004). In line with this, Notch1, a SI lineage marker (Harada et al., 2006), was down-regulated in mutant SI and appeared to show different subcellular localization. Notch signaling is important for SI cell fate specification and in turn ameloblast differentiation (Harada et al., 2006). However, the molecular mechanisms on how SI layer supports enamel formation has not been elucidated. A crosstalk between Notch1 and Bmp2 has been reported in other systems, such as differentiation of smooth muscle cells and dental follicle cells towards the osteogenic lineage (Shimizu et al., 2011)(Viale-Bouroncle et al., 2014), but this crosstalk was not pursued further as part of this thesis.

The limited phenotype caused by epithelial deletion of *Bmp2* in the current study might come as a surprise, as *Bmp2* is expressed in dental epithelium from the placode stage (E11.5) to the early bell stage (E16.5) (Aberg et al., 1997). The K14-Cre driver used in our study was active in oral epithelium at early stages (E11.5) of tooth development, making inefficient Cre-mediated *Bmp2* deletion an unlikely explanation, though it cannot be formally ruled out. The limited phenotype thus might be the result of redundancy, as other Bmps are co-expressed with *Bmp2* in dental epithelium during early tooth development (Aberg et al., 1997; Graf et al., 2016) or could indicate a limited role of *Bmp2* at these developmental stages.

Various Bmp antagonists and other Bmp molecules have shown to affect development of epithelial structures of the tooth. For example- Noggin (a Bmp antagonist) is important in incisor placode formation, which in turn determines the number of incisors (Munne et al.,

2010). Noggin overexpression in oral epithelium results in loss of molar formation (Plikus et al., 2005) indicating a role for epithelial Bmps in early molar tooth development. Another Bmp antagonist, Follistatin is expressed in the enamel free lingual incisor epithelium (Wang et al., 2004). Follistatin ablated lingual epithelium leads to ectopic enamel formation and its overexpression results in disrupted ameloblast differentiation (Wang et al., 2004). Loss of Bmpr1a in dental epithelium during differentiation stage causes a switch from crown epithelium to root epithelium (Yang et al., 2013). These studies are suggestive of a fine balance of Bmp and Bmp antagonists in tooth epithelium important in proper formation of tooth structure. In summary, we have revealed a limited role of epithelial *Bmp2* in organization of the SI epithelial layer leading to a delay in enamel mineralization and possibly enamel quality.

7.2 *Bmp2* regulates reciprocal E-M interaction through WNT signaling and Pax9

Wnt1-Cre is a well-established Cre-driver that targets all neural crest cells (Echelard et al., 1994). *Bmp2* deletion in all neural crest cells did not affect formation of neural crest cells *per se*, but resulted in various craniofacial malformations, including cleft palate with non-complete penetrance, changes to cranial bones, a shorter mandible, and perinatal lethality. These phenotypic changes were at large compatible with the *Bmp2-lacZ* expression and in line with reported phenotypic changes. Wnt1-Cre driver has been widely used for studies on early tooth development but there are no reports on using Wnt1-Cre for later stages of tooth development. As Wnt1-Cre on its own has been shown to have some effect on brain development (Lewis AE et al 2013) the question arises whether the Cre-driver also affects tooth development. As conditional deletion of Bmp2, Bmp7, and Gremlin1 with Wnt1-Cre all cause different phenotypes (this study, Merchant and Graf, Kabani and Graf, unpublished), and no tooth phenotype has been observed in Grem1: Wnt1Cre mutants, such an effect has been considered unlikely.

Deletion of *Bmp2* in dental mesenchyme resulted in dentin and odontoblast differentiation defects, which were first detected at P0. Notably, *Bmp2* was not expressed in dental mesenchyme prior to E18.5. At P0, pSmad1/5/8 staining was overall reduced in mutant teeth, with a reduction observed both in mesenchyme and epithelium. Mesenchymal *Bmp2* thus not only affects dental mesenchyme, but also dental epithelium via epithelial-mesenchymal interactions. qRT-PCR and RNA seq data supported this notion: various oral epithelial and mesenchymal genes were deregulated in mutant teeth including several genes of the Bmp signaling pathway, Wnt signaling pathway, transcription factors and genes involved in dentin and enamel mineralization. Of particular interest was reduced Dentin sialoprotein (*Dspp*) expression and Amelogenin (*Amlx*) expression in the mutants. Mutations in human DSPP (Maciejewska et al 2012) and AMLX (Aldred et al 2003) result in dentinogenesis imperfecta phenotype and amelogenesis imperfecta phenotype respectively. This suggests that mesenchymal Bmp2 might hold a critical role for both dentin and enamel formation. Indeed, conditional deletion of *Bmp2* in mesenchymal cells using *Sp7*-Cre (S. Chen et al., 2009) (Hirata et al., 2009) resulted in enamel defects reminiscent of an amelogenesis imperfecta-like phenotype (Guo et al., 2015).

To understand the earliest events controlling odontoblast and ameloblast differentiation and thus tooth mineralization we focused on elucidating molecular changes at P0 in Bmp2-mutant teeth. Several reports have shown the importance of Wnt/β-catenin signaling for odontoblast differentiation (J. Chen et al., 2009)(Kim et al., 2011)(Kim et al., 2013)(Bae et al., 2015). qRT-PCR on *Bmp2* mutant teeth revealed deregulation of various Wnt pathway genes in both mesenchyme and epithelium *(Wnt3a, Axin2, Lef1, Dkk1, Sost, Sostdc)*. An overall decrease of Wnt signaling was observed when staining for non-phosporylated β-catenin (Amit et al., 2002). This was in line with a reduction in several Wnt-target genes, such as *Lef1, Axin2*. In odontoblasts reduction of Wnt signaling was strongest in areas adjacent to *Bmp2* expressing domains. In the epithelium, non-phosphorylated β–catenin appeared less homogenous but more patchy throughout the ameloblast layer. Expression of

the Wnt inhibitor *Dkk1* was specifically increased in the epithelium of mutant teeth, though *Dkk1* was expressed both in epithelium and mesenchyme as shown in earlier studies (Fjeld et al., 2005) (Suomalainen and Thesleff, 2010). Postnatal deletion of *Dkk1* in mouse molars affects only mesenchyme-derived structures such as dentin and periodontium (Han et al., 2011), suggesting that Dkk1 is an important factor in dentin formation. A role for Dkk1 in epithelial-mesenchymal interactions has been shown during soft palate formation, where upregulation of Dkk1 in the epithelium inhibits Wnt signaling in palatal mesenchyme causing muscle defects (Iwata et al., 2014). This opens the possibility that the effect of *Bmp2*-deletion on mesenchymal Wnt-signaling is indirect via reciprocal epithelial-mesenchymal signaling via Dkk1 rather than direct signaling between neighbouring odontoblasts. Wnt signaling is also important for root formation and periodontal homeostasis (Kim et al., 2013)(Lim et al., 2014)(Bae et al., 2015). Epithelial-derived HERS cells express *Bmp2* and *Bmp2* is persistently expressed in periodontal ligament of adult teeth (Figure 6.1). It is thus possible that a similar crosstalk also exists in other parts of the tooth. However, epithelial *Bmp2*-deletion showed no apparent effect on HERS cells, which might be due to compensation by other Bmps, such as Bmp7, which is also expressed in HERS (Merchant and Graf, unpublished observations). The perinatal lethality of Bmp2fl/fl; Wnt1Cre mutant mice prevented assessing possible effects of mesenchymal *Bmp2*-deletion on periodontal ligament. Therefore, a more specific targeted deletion of *Bmp2* in tooth mesenchyme and tooth epithelium would be apt in conducting tooth developmental studies to study postnatal effects of early Bmp2 deletion in tooth. But, currently these tools are lacking, in terms of the transgenic Cre lines, which would efficiently target tooth specific epithelium and tooth specific mesenchyme cells from early stages. It has been reported that *Pitx2* is specifically expressed in tooth epithelium and *Barx1* in tooth mesenchyme. Designing Cre lines for *Barx1* and *Pitx2* would be important for better understanding of tooth developmental studies.

Nonetheless, the current study provided insights into the changes occurring at the initiation stages of mineralization. Various reports have shown important role for Dkk1 (Li et al.,

2011)(Li et al., 2006)(Morvan et al., 2006) and Bmp2 in bone development and repair (Rosen, 2009)(Pogue and Lyons, 2006)(Carreira et al., 2014). Transcriptional regulation of Bmp2 in skeletal homeostasis involves various signaling pathways including Hedgehog and Wnt/β-catenin signaling (Ran Zhang et al., 2013)(Rongrong Zhang et al., 2013) (Feng et al., 2003) (Garrett et al., 2003) (Zhao et al., 2009). Our study did not analyze Hedgehog pathway, thus the possibility of a cross talk between Bmp-Hedgehog pathway in tooth development cannot be ruled out. Overall our molecular data suggests that *Bmp2* is an important signal for Wnt-mediated odontoblast differentiation and early tooth mineralization is regulated by an intricate crosstalk between Bmp and Wnt signaling possibly involving reciprocal epithelial-mesenchymal interactions involving Dkk1.

Overall decrease in Bmp signaling pathway was also observed as assessed by reduced pSmad 1/5/8 signaling in the mutants, highlighting an important role of *Bmp2* in regulation of the Bmp pathway. Gene expression analysis of various Bmp ligands and antagonists in the mutants revealed interesting insights. *Follistatin*, a Bmp antagonist involved in enamel formation (Wang et al 2004) was greatly reduced in the mutants whereas *Bmp4* was upregulated. Initially this pointed towards a compensatory mechanism of *Bmp4* in *Bmp2* mutants. However the separation experiment (Figure 6.23 C) ruled out this possibility as Bmp4 was upregulated in the epithelium. Therefore it is important to consider studying expression analysis in specific tooth tissue type (epithelium or mesenchyme) as opposed to analyzing the whole tooth structure. Gene expression analysis on whole tooth might lead to oversimplification of the data.

qRT-PCR analysis also revealed changes to the expression of several transcription factors with known important roles during in early tooth development or tooth mineralization such as *Pax9, Msx1, Tbx1* (Zhao et al., 2013). *Pax9* is expressed in mesenchyme at early stages of development (E10.5) and its absence leads to tooth agenesis (Peters et al., 1998). *Pax9* expression is induced by oral epithelium-derived Fgf8 (Neubüser et al., 1997). PAX9 in humans causes selective tooth agenesis (Stockton et al., 2000)(Nieminen et al., 2001). In

our study, we find both mesenchymal and epithelial expression of *Pax9*. In the absence of *Bmp2,* Pax9 was greatly reduced in ameloblasts suggesting that mesenchymal *Bmp2* affects Pax9 protein expression. In contrast to this, qRT-PCR on isolated tooth epithelium revealed increased expression of *Pax9*. This discrepancy indicates that *Bmp2* signaling regulates Pax9 mRNA translation rather than gene transcription. A role of Bmp signaling on protein translation has to our knowledge not been reported. Protein translation is highly regulated, for example via regulation of RNA binding protein, control of initiation factor activity and regulation by micro-RNAs (for detailed review see (Jackson et al., 2010). More detailed studies are needed to elucidate how Bmp2 affects Pax9 protein translation. Controlling protein translation as a mean to control development has been observed in other developmental processes: mutations in various forms of protein translation regulators has been observed in various forms of autisms (Yang et al 2014).

Tbx1 is expressed in the enamel-forming labial incisor epithelium but not in the lingual epithelium. Ectopic expression of *Tbx1* in the lingual incisor epithelium correlates with Amelogenin expression and mice lacking *Tbx1* fail to form enamel (Catón et al., 2009). Loss of mesenchymal *Bmp2* affects induction of *Tbx1* at P0, as well as several genes associated with enamel formation (*Amlx, Abn, Mmp13*). The reduced levels of pSmad1/5/8 observed in mutant molar epithelium thus indicate that *Bmp2* is a critical early signal for ameloblast differentiation and enamel formation. Currently, it is not clear if and how *Tbx1* interacts with *Pax9*, and whether this induction is direct or indirect.

Overall, our gene expression analysis at P0 has revealed gene networks regulated or affected by mesenchymal *Bmp2* expression. Loss of *Bmp2* affects odontoblast differentiation and the Wnt pathway in the mesenchyme and reciprocal epithelial-mesenchymal interactions regulating ameloblast differentiation and enamel formation via Bmp signaling, Pax9 and Tbx1.

Two studies performed alongside ours has confirmed that *Bmp2* is important for odontoblast differentiation and pulp vascularization (Yang et al., 2012) as well as development of

periodontal tissue (Rakian et al 2013)., In their studies the authors used the Col1a1-Cre, which is not active in odontoblasts prior to P1 (Gluhak-Heinrich et al., 2010), a time point slightly later than when the onset of our tooth mineralization phenotype was observed. As mentioned earlier, the same group also reported enamel defects in these *Bmp2*-mutant mice (Guo et al 2015). No detailed molecular or cellular analysis was performed for either of these studies. As *Bmp2*-deletion in our study occurred at embryonic stages in all neural crest cells, we were able to catch earliest effects on tooth mineralization.

7.3 *Bmp2* is important in homeostasis of the tooth pulp

To understand the role of *Bmp2* in adult teeth we transplanted *Bmp2*-deficient tooth germs under the kidney capsule of the recipient mice. 4 week *in situ* grown mutant teeth showed various morphological changes: reduced thickness of the dentin layer, poorly organized dentin tubules, and ectopic mineralization of the pulp, which commonly referred to as pulp obliteration. Pulp obliteration is seen in some forms of dentinogenesis imperfecta: Dentinogenesis imperfect type II caused by mutations in *Dspp*, one of the genes downregulated in *Bmp2*-mutant teeth; shell teeth, termed after their thin dentin layer; dentin dysplasia. Pulp obliteration is also associated with tooth ageing or trauma due to injury or periodontal disease (Goga et al., 2008). It will be of interest to investigate whether deregulation of *Bmp2* is associated with pulp obliteration under those circumstances, in particular since expression of *Bmp2* persists in the pulp of adult teeth. Histochemical analysis of obliterated teeth revealed an overall decrease in pSmad1/5/8 and Dsp. The decrease in Bmp signaling was observed in tooth root, periodontium, alveolar bone and pulp. Possibility of heterodomerization of *Bmp2* along with other Bmps cannot be ruled out (Bmp2/4, Bmp2/7) (Hamaratoglu et al., 2014)(Brazil et al., 2015b). Unpublished reports from our lab have shown that adult *Bmp7* deficient mice show pulp and dentin defects with altered Bmp signaling. It might be suggestive of various Bmp molecules taking part in the homeostatis and maintainance of the tooth structure during development and in adult tooth.

A similar pulp calcification phenotype was noted in an article published parallel to our work (Rakian et al., 2013), though this phenotype was not further addressed. This report also showed that *Bmp2* is also important for tooth root and periodontium development. Rakian et al used SP7-Cre, which is expressed in the odontoblasts at P0, making their model viable however not appropriate to analyze early *Bmp2* deletion effects. Here we show that *Bmp2* is an important factor important for pulp homeostasis.

7.4 Interlinked signaling feedback loops: Insights from other organ systems

Most of the organs arising through E-M interactions (for example teeth, lung, kidneys, salivary gland, hair follicle and mammary gland) use similar molecular signals during early stages of their development, such as Bmp, Hh, Wnt, Fgf signaling. However they arise from different embryonic germ layers: For example the mesenchyme component of hair follicle arise from mesoderm layer (Lavker et al., 2003) and mammary glands arise from epidermal endoderm (Sreekumar et al., 2015). As tooth arises from oral ectoderm and neural crest-derived mesenchyme (which is a specialized mesenchyme capable of differentiating also into more cell types than a mesodermal derived mesenchyme), the question to consider is whether results from this study are comparable or even transferable to other developmental systems. It is clearly important to consider these factors when interpreting results in developmental systems. Nevertheless current knowledge from other systems, such as limb bud organogenesis can be utilized, as the early signals are similar in tooth and limb buds. It is fair to assume that protein-protein interactions are independent of the tissue in which they occur. However, co-factors, genetic (via enhancers/silencers) and epigenetic regulation will affect the outcome of these interactions. To that effect, significant similarities between limb and tooth development can be identified. Initial experiments performed in identifying the morphogenesis of limb buds focused on a single gene or a single pathway, but studies these days show limb organogenesis is controlled by a interlinked feedback loops instead of an independent morphogen signals (Zeller et al 2009), similar to what was observed for tooth

mineralization in this study. In the limb, members of the FGF family are important in specification of apical epithelial ridge (AER). Disruption of Fgf10 and Bmp4 in mouse AER disrupts formation of limb buds. An early epithelial-mesenchymal feedback loop exists between Fgf8 and Fgf10 in early growth of limb buds (Ohuchi et al., 1997). Fgf pathway, which is also important in the initial stages of tooth development, but was not studied in the current project because it did not appear prominent in the RNAseq experiment. Additional studies are necessary to formally asses this. Shh is another important signal whose graded activity in mouse bud limb development is required for development. Shh upregulates or induces expression of target genes such as Gli1 and Patch1 receptor (Yang et al., 1997). Processing of Gli3 to its repressor form is inhibited in response to SHH activity; whereas the full-length activator form act as positive transcriptional regulator (Wang et al., 2000). The anterior limb bud contains high Gli3 repressor form and low full-length activator form. In the posterior region of the limb bud, close to Shh signaling activity the opposite is true. Human and mouse mutations of Gli3 have shown polydactylies. However these mutations only affects digit 2 and recent studies show that only digit 2 depends entirely on SHH signaling. An early SHH-GREM1-FGF E-M feedback loop is important for apical epithelial ridge in limb formation (Michos et al., 2004)(Zúñiga et al., 1999). Grem1, a Bmp antagonist deletion in mouse limb bud has indicated that GREM1- mediated reduction of BMP is important for limb bud development. Mutations in Grem-2 in mice have also been shown to affect incisor teeth (Vogel et al., 2015), and roles for several Shh target genes in tooth development have been shown (Matalova et al 2008; Nanni N et al 2001)

Thus, studies in organ system, which arise through E-M interaction, give us an insight into the molecular mechanisms that might be involved in a variety of developmental systems arising through E-M interactions. However, there are clear differences in the cell types which gives rise to specialized structure making each organ system unique. The challenge is to appropriately consider these differences while comparing the various organ systems. One obvious difference would be epigenetic regulation, which is most commonly mediated by

histone modification and DNA methylation (Jaenisch et al 2003).and which directly affects gene expression.. However, protein-protein interactions are expected to remain the same irrespective of the cell type, provided there are no post-translational modifications or presence of modifier proteins. Thus, whereas should be possible to draw some general conclusion from every developmental system, the challenge is in distinguishing general and tissue-specific interactions.

7.5 Challenges to interpret complexity of developmental systems

The developing tooth forms its various specialized structures such as enamel, dentin, root, periodontal ligaments. Partly simultaneously these structures arise through various cells types. For example odontoblasts, which give rise to dentin undergo a series of differentiation stages, which are all present in the development tooth. The most mature odontoblasts residing in the crown region of the tooth give rise to dentin. These odontoblasts are elongated and have a palisade structure with distinct morphology from other differentiating odontoblasts. However the odontoblasts lining the root dentin are more flat and gives rise to root by interacting with HERS cells. This interaction is different from the odontoblasts and ameloblast interaction that give rise to dentin and enamel respectively (Nanci 2013). When studying gene expression it is important to correlate molecular changes to the various differentiation stages. Currently, there is a lack of information regarding the signals that would define precursor odontoblast cells, preodontoblast and odontoblasts (Nanci 2013). However, this knowledge is critical if we want to understand the intricacies of tissue development. A suitable method for understanding developmental processes in detail would be single cell transcriptomics. This method has recently been applied to lung development, an organ which also arises through E-M interaction (Robson, 2014). In this study the authors used microfluidics to capture individual cells from which amplified cDNA was produced (Treutlein et al., 2014). Recently the sensitivity of single cell RNA-seq was

improved making it amendable for this type of studies. (Wu et al., 2014). Although there are caveats to these systems single cell transcriptoms were generated from an E18.5 mouse fetal lung and five distinct populations of cells were identified. This kind of transcriptome wide analysis provides new cell type specific markers that are critical for future developmental genetic studies (Robson P et al, 2014). Such a system could be employed for tooth developmental studies to distinguish and identify odontoblast subpopulations representing various maturation stages or individual cell populations giving rise to dentin and root respectively. In addition this would provide an insight into specific markers of odontoblast at the various differentiation stages.

In summary, the current study utilized tooth as a model to study E-M interaction in the context of Bmp2. Various roles for Bmp2 in tooth organogenesis were demonstrated. Though knowledge from this study will be transferable to other developmental systems, it is important to consider the factors described above for proper interpretation as well as the complexity of the various systems.

8. Abbreviation

ALK- Activin Like Kinases

Am-ameloblasts

Amlx- Amelogenin

cDNA-complementary- DNA

Cont-Control

CNCC-Cranial Neural Crest Cells

cKO-conditional Knock out

AER-Apical Epithelial ridge

BMP- Bone Morphogenetic protein

BMPR1-BMP receptor

BCIP-5-Bromo-4-Chloro-3-Indolyl Phosphate

DIG-digoxygenin

DSPP- Dentin sialoprotein

DMP-Dentin Matrix protein

DNA-Deoxyribonucleic Acid

D-Dentin

ECM-Extracellular Matrix

Epi-Epithelium

En-Enamel

E-M-epithelial mesenchymal

FGF-Fibroblast Growth Factor

FGCZ-Functional Genomics Centre Zurich

GREM-Gremlin

Hh- Hedgehog

HERS-Hertwig`s epithelial root sheath

ISH- Insitu Hybridization

IHC-Immunohistochemistry

In-Incisors

KOH-Potassium hydroxide

KCT-Kidney Capsule Transplantation

liCL- Lingual Cervical Loop

laCL-Labial Cervical Loop

M-E- Mesencymal Epithelium

$MgCl_2$-Magnesium Chloride

Mes- mesenchyme

MMP- metalloproteinase

Mut-Mutant

Micro-CT- Computed Tomography

M1,M2,M3-Molar 1,2,3

NBT-Nitroblue tetrazolium

NaCl-Sodium Chloride

NCC-Neural crest cells

Od-Odontoblasts

Oe-oral epithelium

OC-Osteocalcin

OPG-Osteoprotegrin

Osx-Osterix

Pax9-Paired box gene

Pdl- Periodontal Ligament

Pd-Periodontium

PBS- Phosphate Buffered Saline

PFA-Paraformaldehyde

PN-Postnatal

P0-Postnatal day 0

PCR-Polymerase Chain reaction

pSMAD-phospho-SMAD

RT-PCR-Real time Polymerase chain reaction

RNA-Ribonucleic acid

SI- Stratum Intermedium

SHH-Sonic Hedgehog

TGF-Transforming Growth Factor

t- tongue

VDR-Vitamin D receptor

9. References

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10. Curriculum Vitae

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Educational and research experience

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January 2012-June 2016 : Ph.D. studies
"Role of Bmp2 in dental hard tissue formation"
University of Zurich, Switzerland

September 2009–August 2010: Master of Sciences (M.Sc.) in Molecular Medicine
University of Sheffield, England, UK

January 2011-October 2011: Research Assistant in Department of Molecular Medicine
University College London, London, UK

2009-2010 Master thesis
"Role of XMRV in prostate cancer"
Department of Bone Biology, University of Sheffield, UK

2006–2009 Bachelor of Sciences (B.Sc.)
Lucknow University, Lucknow, India

Research Publications

Common mechanisms in development and disease: BMP signaling in craniofacial development
Daniel Graf · **Zeba Malik** · Satoru Hayano · Yuji Mishina
Nov 2015 · Cytokine & growth factor reviews

Teeth from Noggin-deficient mice reveal significance of topical BMP signaling for tooth and periodontal development
Minglu Ye · **Zeba Malik** · Maria Alexiou · Thimios A. Mitsiadis ·Daniel Graf
Conference Paper · Jul 2015

Altered BMP signaling in the dental pulp leads to dentin dysplasia with pulp obliteration
Zeba Malik · Anastasiia Kozlova · Maria Alexiou · Thimios A. Mitsiadis · Aris N. Economides · Daniel Graf
Conference Paper · Jul 2015

Selective Scientific presentations, posters

- **2016** Poster presentation and 60 second science talks at Canadian Developmental Biology Meeting, Banff, Canada
- **2015** Poster Presentation "75th Developmental Biology Meeting", Utah, US
- **2014** Poster presentation at Bmp conference, Berlin, Germany
- **2013** Poster presentation at "Tooth Morphogenesis and Differentiation conference, Nice, France
- **2013** Stem cell network, Bern, Switzerland

Grants and fellowships

- **2010** Department of Medicine, University of Sheffield, UK 2000 pounds Scholarship for pursuing M.Sc. in Biotechnology at University of Sheffield, UK

Organization and leadership skills

- **Public Speaking**- Participated in Three minute thesis competition at University of Alberta, Alberta, Canada

- **2012 Student representative** in interviews at Molecular Life Sciences, Zurich

- **Supervision** of two undergraduate students and one masters student in research projects.

- **Participation in organizing and supervising** Bio111 (practical lab course) of undergraduate students of Biology at University of Zurich in 2012 and 2013

Languages

Hindi (fluent in oral and written)
English (fluent in oral and written)
German (Basic)
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